IDENTIFICATION AND CHARACTERIZATION OF THE UL37 PROTEIN OF HERPES SIMPLEX VIRUS TYPE 1 AND DEMONSTRATION THAT IT INTERACTS WITH ICP8, THE MAJOR DNA BINDING PROTEIN OF HERPES SIMPLEX VIRUS

1992

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APPROVAL SHEET

Title of Dissertation: "Identification and Characterization of the UL37 Protein of Herpes Simplex Virus Type 1 and Demonstration that It Interacts with ICP8, the Major DNA Binding Protein of Herpes Simplex Virus"

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Doctor of Philosophy Degree
October 20, 1992

Dissertation and Abstract Approved:

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Committee Member	12-30-92 Date
Committee Member	10-20-92 Date
Committee Member	10-20-92 Date
Committee Member	10-20-92 Date

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ABSTRACT

Title of dissertation: Identification and characterization of the UL37 protein of herpes simplex virus type 1 and demonstration that it interacts with ICP8, the major DNA binding protein of herpes simplex virus.

Lisa S.G. Shelton, Doctor of Philosophy, 1992

Dissertation directed by: Frank Jenkins, Ph.D., Assistant Professor, Department of Microbiology

We have identified and characterized the previously unknown protein encoded by the UL37 gene in HSV-1-infected cells. Using *in vitro* transcription and translation of the UL37 gene, we have generated a UL37-specific antiserum to identify the UL37 protein in HSV-1-infected cells. The UL37 protein has an apparent molecular weight of 120 kD based on its migration on SDS-polyacrylamide gels and is indistinguishable by immunoblot analysis from the UL37 protein expressed by a vaccinia recombinant. By time course experiments and use of a DNA synthesis inhibitor, we have demonstrated that the UL37 protein is expressed prior to the onset of DNA synthesis, but requires viral DNA synthesis for maximum expression. Based on these results, we have classified the UL37 gene as belonging to the γ1 class of HSV genes. Analysis of purified virions failed to detect the UL37 protein, demonstrating that it is a nonstructural

protein.

Analysis of HSV-1-infected cell proteins by single-stranded and double-stranded chromatography demonstrated that the UL37 protein exhibits a high affinity DNA binding activity, co-eluting with the HSV-1 major DNA binding protein, ICP8. ICP8 is an essential, multifunctional viral protein, involved in viral DNA replication and late gene regulation. We have compared the DNA binding activities and nuclear localization of the UL37 proteins derived from cells infected with HSV-1, an HSV-1 recombinant (d21) containing a deletion in the ICP8 gene, and a vaccinia recombinant containing the UL37 gene. These studies demonstrated that the expression of UL37 in the presence of a functional ICP8 protein is required for the observed DNA binding activity and nuclear localization of the UL37 protein in HSV-1-infected cells. Therefore, the UL37 and ICP8 proteins form a complex in HSV-1-infected cells.

To address the functional role for the UL37-ICP8 complex in HSV replication, several mutations in the UL37 gene were constructed to generate an HSV recombinant deficient in UL37 expression. Based on the expression of UL37 late in HSV infection, we postulate that the UL37 protein assists or modifies the ICP8 protein, affecting its ability to function in DNA replication and/or late gene regulation.

Identification and Characterization of the UL37 protein of Herpes Simplex Virus Type 1 and Demonstration that It Interacts with ICP8, the Major DNA Binding Protein of Herpes Simplex Virus

by

Lisa S.G. Shelton

Dissertation submitted to the Faculty of the Department of Microbiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1992.

TO GOD BE THE GLORY

ACKNOWLEDGEMENTS

I would like to thank the members of the Department of Microbiology for their encouragement and special thanks to my committee for their time, guidance, support, and patience during the preparation of this dissertation.

Thanks to all the graduate students who I've been privileged to know and who have shared this experience with me.

To Drs. Kathryn Holmes and Stefanie Vogel for their special encouragement and support to me as a female scientist.

Special thanks to the members of the Jenkins and Hay labs, who got me started in the lab and shared their expertise with me: Alyson Chapman, Dr. Paul Kinchington, Dr. Michael Pensiero, Michael Flora, Paul Ling, and Jace Hougland. Also, thanks to Pam Glass, Jon Hirsch, Darrell Griffin, Marie Hall, Wannee Kantakamalakul, and Allen Albright for their constant support and friendship.

I would like to especially thank Dr. William Ruyechan and his lab for their valuable guidance and collaborative efforts.

To Dr. Frank Jenkins, my utmost appreciation for training, guidance, availability, understanding, and unending patience. This has truly been a learning experience.

Most importantly, to my husband for his support, encouragement, understanding, and tremendous respect for my desire to accomplish such an immense, and at many times overwhelming, project; to our parents, family, and friends for their constant love, support, and prayers.

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INTRODUCTION

Herpes simplex virus is a member of the *herpesviridae* family and the subfamily alphaherpesvirinae (Roizman *et al*, 1981). Membership in the *herpesviridae* family is based on a virion architecture consisting of four structural components: (1) a core containing a linear, double-stranded DNA in the form of a torus, (2) an icosahedral capsid, 100-110 nm in diameter, comprised of 162 capsomeres, (3) a globular material surrounding the capsid referred to as the "tegument", and (4) an envelope containing surface glycoproteins (Matthews, 1982)(Fig.1). As a family, these viruses are indistinguishable by electron microscopy and share distinct biological properties including a nuclear location for viral DNA synthesis and capsid assembly and the ability to remain latent in their natural hosts.

Herpesviruses have been classified into three subfamilies based on differences in biological properties which are believed to be useful in determining evolutionary relatedness (Roizman *et al*, 1981). The human alphaherpes-viruses including herpes simplex viruses type 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV) display a variable host range (from very wide to very narrow), a relatively short reproductive cycle, rapid spread in cell culture resulting in efficient destruction of susceptible cells, and the capacity to establish latent infections frequently, but not exclusively, in sensory ganglia. Other

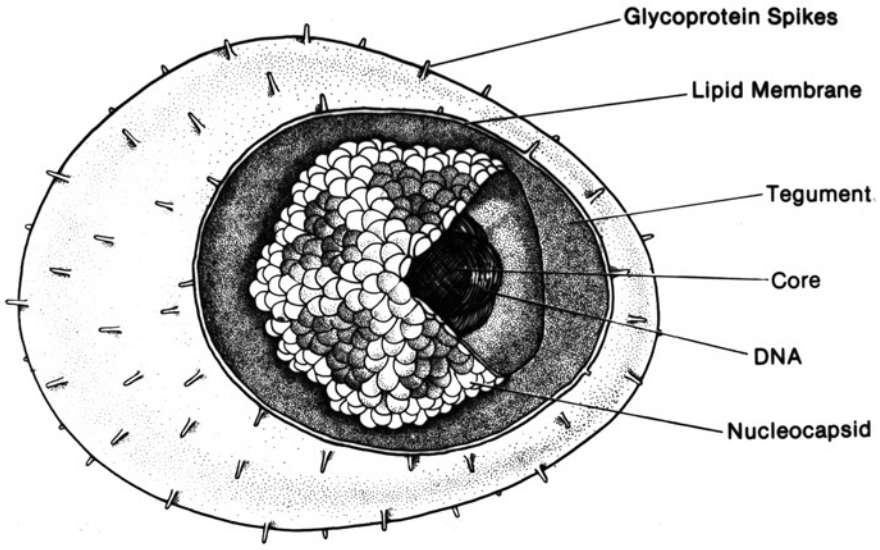


FIGURE 1

Structural model of the herpesvirus virion. A cutaway diagram of a virion showing the individual structural components. (Reproduced with permission from Hay *et al*, 1987)

human herpesviruses include human cytomegalovirus (beta-herpesvirus), Epstein-Barr virus (gammaherpesvirus), and human herpesviruses 6 and 7 (unclassified). These herpesviruses differ greatly with respect to the clinical syndromes they cause and their genomic structures.

HSV INFECTIONS

Common infections

HSV infection results in a variety of clinical manifestations, ranging from mild to life threatening, depending upon the route of infection and the age and immunologic status of the host (reviewed in Nahmias and Roizman, 1973; Corey and Spear, 1986; Whitley, 1985; White and Fenner, 1986; Whitley, 1990; and Peterslund, 1991) (Table 1). Infections can occur by contact with an infected individual or by autoinoculation. Primary infection with HSV most commonly involves virus contact with the mucous membranes and skin of either the oropharynx, the genitalia, or the eye (Dodd *et al*, 1938; Gallardo, 1943). Following primary infection, a hallmark of herpesvirus infection is the ability to establish latent infection which may appear as recurrent infection throughout the lifetime of the infected individual (Burnet and Williams, 1939; Scott *et al*, 1941). HSV-1 and HSV-2 infections are usually transmitted by different routes, involve different areas of the body, and are commonly referred to as "above-the-waist" and "below-the-waist" infections, respectively (Whitley, 1985).

TABLE I

Diseases Produced by Herpes Simplex Virus

DISEASE	PRIMARY (P) OR RECURRENT (R)	AGE	FREQUENCY	SEVERITY	TYPE
Gingivostomatitis	P	Young children	Common	Mild	1
Pharyngotonsillitis	P	Adolescents	Common	Mild	1 > 2
Herpes labialis	R	Any	Common	Mild	1 > 2
Genital herpes	P, R	>15 years	Common	Mild to moderate	2 > 1
Keratoconjunctivitis	P, R	Any	Common	Moderate	1
Dermatitis[a]	P, R	Any	Rare	Mild	1, 2[b]
Encephalitis	P, R	Any	Rare	Severe[c]	1 > 2[d]
Neonatal herpes	P	Newborn	Rare	Severe[c]	2 > 1
Disseminated herpes	P, R	Any	Rare	Severe[c]	1 > 2

[a] Including HSV infection of burns, eczema herpeticum, etc.
[b] Skin above waist, 1 > 2; below waist, 2 > 1; arms, either.
[c] Often fatal.
[d] HSV-2 in neonates.

(Reproduced with permission from White and Fenner, 1986)

While the predominance of HSV infections differ in their distribution between these two routes of infection (Nahmias and Dowdle, 1968), both serotypes are capable of infecting both anatomical sites, and the pathogenesis of the viruses appears to be similar.

Pathogenesis

Our current understanding of the pathogenesis of recurrent oral, genital, and ocular HSV infections results from studies in experimental animals coupled with clinical observations (reviewed in Hill, 1985; Whitley, 1985; Corey and Spear, 1986; Stanberry, 1986; Whitley, 1990)(Fig.2). Briefly, viral replication at the site of infection such as mucosal surfaces, corneal epithelium, or abraded skin, causes vesicles and ulcerations lasting 2-3 weeks, but often results in no obvious symptoms. Following the initial infection, the virus capsids are transported by retrograde axonal flow to the peripheral ganglion responsible for innervating the site of local replication (Cook and Stevens, 1973; Kristensson *et al*, 1971; Kristensson *et al*, 1986). For oral and genital HSV infections, this corresponds with the trigeminal and sacral ganglia, respectively (Baringer and Swoveland, 1973; Baringer, 1974). Once the virus reaches the ganglion, a latent infection is established (Stevens and Cook, 1971). Despite recovery from the primary infection, the infected individual retains HSV DNA in the ganglion for life and may suffer recurrent attacks of herpes

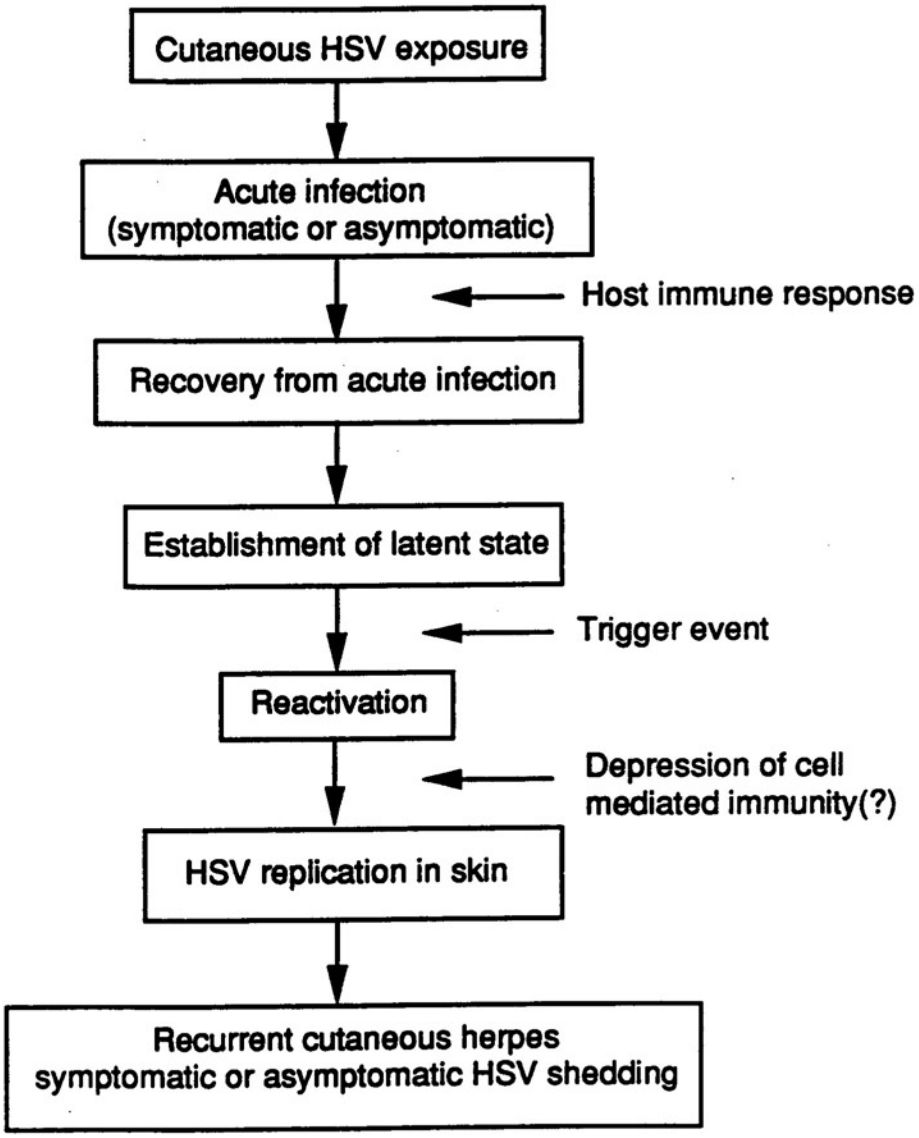


FIGURE 2

Schematic diagram of HSV pathogenesis. Events involved in the pathophysiology of recurrent HSV infection. (Reproduced with permission from Stanberry, 1986)

labialis ("cold sores" or "fever blisters") or genital herpes as a result of virus reactivation (Baringer and Swoveland, 1973; Baringer, 1974, Baringer, 1976). Paradoxically, recurrences occur in the presence of humoral immunity (Andrewes and Carmichael, 1930), and reinfection with different strains of HSV can occur, albeit rarely (Buchman *et al*, 1979). Several external stimuli and immunosuppression have been implicated in triggering reactivation of latent HSV (Greenberg *et al*, 1969; Segal *et al*, 1974; Baringer, 1976; Whitley, 1984; Greenberg *et al*, 1987); however, the exact mechanisms involved remain unclear.

The HSV replication cycle

Latent infection *in vivo*

HSV causes lytic infection in epithelial cells at the site of viral inoculation (Cook and Stevens, 1973; Leib *et al*, 1989a). The virus spreads, infecting sensory neurons that innervate the area of infection, and the nucleocapsid is transported axonally by retrograde flow to the nerve cell body in the sensory ganglion (Cook and Stevens, 1973; Kristensson *et al*, 1971; Kristensson *et al*, 1986)(Fig.3). Once the virus reaches the neuronal nuclei of the sensory ganglion, the virus is capable of replication for a number of days (Knotts *et al*, 1974; Steiner *et al*, 1989) which can be detected in cell-free homogenates of ganglionic tissue (also known as acute infection).

Following the acute infection, a latent infection is

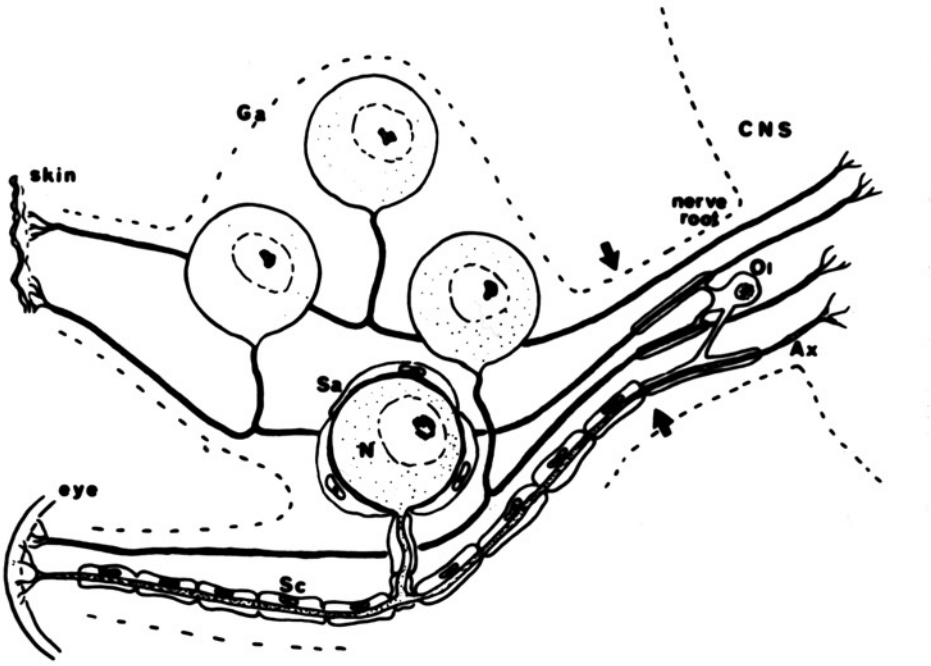


FIGURE 3

Diagram of the main anatomical features of a sensory ganglion and peripheral nerve. Only one neuron is shown in full detail. Ga, ganglion; Sa, satellite cell; Sc, Schwann cell; N, neuron; Ol, oligodendroglial cell; Ax, axon; arrows, junction of PNS and CNS. The diagram is particularly illustrative of the ophthalmic part of the trigeminal (fifth cranial nerve) ganglion, which supplies sensory nerves to the eye and some areas of the skin of the head. (Reproduced with permission from Hill, 1985)

established in the ganglion which can be detected by reactivation in vitro by an explantation and co-cultivation technique developed by Stevens and Cook (1971)(reviewed in Baringer, 1975; Baringer, 1976; Stevens, 1976; Hill, 1985; Stanberry, 1986; Stevens, 1989). A latent infection is operationally defined as one where infectious virus can be isolated from explanted tissue (intact ganglia), but not from cell-free tissue homogenates (Hill, 1985; Stanberry, 1986). It appears that *in vitro* culture of the explant allows for reactivation of the virus which can subsequently be detected by co-cultivation with permissive cells (Stevens and Cook, 1971).

Two theories exist regarding the establishment of latent infection within the ganglion: (1) the virus replicates in some neurons resulting in their destruction and establishes latency in others (presumably due to an abortive infection) which are detected by reactivation from ganglionic tissue, or (2) the virus replication within a given neuron proceeds to a limited degree, but the permissiveness of the infected neuron is transient, resulting in the establishment of a latent state. In either case, the cellular restrictions on viral replication which result in latency have not yet been identified. In addition, studies with HSV mutants indicate that viral replication within the neuronal nucleus is not necessary for establishment of latent infection or reactivation (McLennan

and Darby, 1980; Steiner *et al*, 1990). Whether viral replication either during establishment of latency or during reactivation results in neuronal destruction continues to be debated (Klein, 1976; McLennan and Darby, 1980; Stevens, 1989; Roizman and Sears, 1990; reviewed in Hill, 1985).

In latently infected cells, the viral genome can be detected by Southern hybridization (Rock and Fraser, 1983), and the DNA exists as a circularized molecule (Rock and Fraser, 1985; Mellerick and Fraser, 1987). Latent HSV, due to its nonreplicative state, is unaffected by treatment with antiviral drugs which inhibit viral replication (Wohlenberg *et al*, 1976; Field *et al*, 1979; Park and Pavan-Langston, 1982). At least three herpes-specific latency-associated transcripts (LATs; 2.0, 1.5, and 1.45 kb), one unspliced and two spliced mRNAs, mapping to the same genomic location, which are partially antisense to one of the HSV regulatory proteins (ICP0), are expressed during latent infection (Stevens *et al*, 1987; Spivack and Fraser, 1987; Rock *et al*, 1987). Their function in latency is unknown since removal of the LAT gene does not alter the ability of the virus to establish latency in experimental animal models (Javier *et al*, 1988; Steiner *et al*, 1989; Leib *et al*, 1989b). The role of LATs in reactivation is still in question since LAT$^-$ mutants are capable of reactivation *in vitro* (Javier *et al*, 1988; Steiner *et al*, 1989; Leib *et al*, 1989b), but some LAT$^-$ mutants show either a prolonged reactivation time (Steiner,

1989) or reactivation at a reduced efficiency (Leib *et al*, 1989b).

Virus-encoded determinants for establishment or maintenance of latency have not been identified, although recent work from the laboratory of Priscilla Schaffer implicated the ICP0 regulatory protein (Leib *et al*, 1989a) and an unidentified cellular ICP0-like protein (Cai and Schaffer, 1991) in reactivation. Several stimuli (physical or emotional stress, fever, menstrual cycles, exposure to UV light, trigeminal root sectioning, immunosuppression, nerve sectioning, and physical or chemical trauma to the innervated peripheral site) can cause reactivation of the viral genome which results in productive infection at or near the original site of infection (Greenberg *et al*, 1969; Segal *et al*, 1974; Carton and Kilbourne, 1952; Greenberg *et al*, 1987; Walz *et al*, 1974; reviewed in Baringer, 1976; Hill, 1985). Whether the reactivation of a latent state to a lytic infection involves suppression of host-supplied factors that render the neuron nonpermissive or changes in neuronal physiology remains unclear.

Lytic infection in cell culture

Although HSV manifests a neurotropism *in vivo* following injection into a peripheral site, in cell culture HSV demonstrates a wide host range, lytically infecting a variety of cells from both animals and humans including those of neuronal origin (Leetsma *et al*, 1969). No cell

culture system exists that allows efficient establishment of latent infection without the use of antiviral inhibitors. Therefore, latent infection must be studied in experimental animals and, as a result, the lytic replication cycle of HSV in cell culture systems has been more extensively studied.

The replicative cycle of HSV in cell culture is rapid, approximately 12-18 hours in duration, with viral eclipse occurring at 5-8 hours postinfection (Roizman and Furlong, 1974). Infection by HSV is initiated by the binding of the enveloped virion to surface receptors of the host cell. Of the nine HSV glycoproteins, gB and gC on the viral envelope are involved in the initial binding to heparan sulfate proteoglycans on the cell surface (Herold *et al*, 1991; Shieh *et al*, 1992; WuDunn and Spear, 1989). At least three HSV glycoproteins (gB, gD, and gH) are essential for entry into the cell (Spear, 1985; Sarmiento *et al*, 1979; Ligas and Johnson, 1988). Most recently, Fuller and Lee (1992) have proposed that a cascade of virus-cell interactions occur which leads to HSV penetration and involves several HSV components with cooperative functions. This proposal is based on the findings that gD and gB are each involved at distinct stages of attachment and fusion which have been defined by electron microscopic studies (Fuller and Lee, 1992). Once the nucleocapsid gains entry into the cytoplasm by fusion of the the viral envelope with the plasma membrane (Para *et al*, 1980; Spear *et al*, 1989), it is then

transported to the nuclear pores where release of viral DNA occurs (Batterson *et al*, 1983).

Transcription, replication of viral DNA, and assembly of new capsids occur in the nucleus (reviewed in Knipe, 1989; Roizman and Sears, 1990). Transcription of viral genes by the host RNA polymerase II proceeds in a cascade fashion, resulting in the differential expression of three classes of genes: immediate early (IE) or α; early (E) or β (also known as delayed early, DE); and late (L) or γ (Honess and Roizman, 1974). These gene classes have been defined by the use of certain metabolic inhibitors. The α genes, by definition, are transcribed very shortly after infection in the absence of any *de novo* viral protein synthesis. A virion-associated protein, the α-*trans*-inducing factor (αTIF, Vmw65, or VP16) is responsible for transactivation of the α gene promoters (Post *et al*, 1981; Campbell *et al*, 1984; Preston *et al*, 1988). Of the five α proteins, at least three regulate later gene expression including ICP4 which is required throughout infection for the maintenance of β and γ gene expression (Watson and Clements, 1980). The β genes encode proteins needed for viral DNA synthesis and enzymes involved in nucleic acid metabolism and, by definition, require functional α proteins for their transcription (Honess and Roizman, 1975). The onset of β gene expression coincides with the decline of α gene transcription. The γ genes are differentiated from β genes

based solely on their requirement for DNA synthesis for maximal expression. The γ genes have been subclassified into two groups, γ1 and γ2, based on their their timing of expression and dependence on viral DNA synthesis: γ1 gene expression is stimulated by, but not absolutely dependent upon, viral DNA synthesis, and γ2 gene expression is completely dependent on viral DNA replication. Most of the γ proteins are structural proteins (Spear and Roizman, 1968).

DNA replication is believed to occur by a rolling circle mechanism since the incoming viral DNA molecule loses its termini (Mocarski and Roizman, 1982) shortly after infection (Poffenberger and Roizman, 1985), presumably by circularizing upon release from the nucleocapsid, and concatermic forms of DNA are observed (Ben-Porat *et al*, 1976; Jacob *et al*, 1979). Although the process of DNA replication is not well understood, three *cis*-acting elements have been identified (ori_L and two ori_S) and a number of β proteins are required (reviewed by Weller, 1991). At least 7 HSV proteins are directly involved in the synthesis of DNA (Olivo and Challberg, 1988). The process of viral DNA synthesis activates the expression of γ genes by an unknown mechanism. Some of the resulting γ proteins are localized to the nucleus where they assemble to form progeny capsids, and some are incorporated into the nuclear and plasma membranes (Spear and Roizman, 1968; Gibson and

Roizman, 1972; reviewed in Dargan, 1986). The viral DNA is cleaved into unit-length molecules from concatamers and packaged into preformed empty capsids. Two separate signals (*pac*-1 and *pac*-2) are responsible for this cleavage/packaging event (Deiss *et al*, 1986). Only nucleocapsids containing full-length or nearly full-length genomes escape from the nucleus (Vlazny *et al*, 1982), presumably due to structural changes of the nucleocapsid (Gibson and Roizman, 1972).

The site of HSV envelopment is generally accepted as the inner nuclear membrane, occurring at dense patches, most likely representing tegument proteins on the inside surface and immature HSV glycoproteins on the outside surface (Darlington and Moss, 1968; reviewed in Spear, 1985; Dargan, 1986; Roizman and Sears, 1990), and such enveloped particles are believed to be transported through the cisternae of the cell including the rough endoplasmic reticulum and Golgi to the plasma membrane (Fig.4). However, there are some reports of herpesvirus envelopment at cytoplasmic membranes, suggesting that virus particles gain and lose envelopes by passing through both the inner and outer nuclear membranes and eventually obtain an envelope by budding into cytoplasmic vacuoles (Stackpole, 1969; reviewed in Dargan, 1986). Although not essential for virion assembly or infectivity, posttranslational modifications for maturation of HSV glycoproteins and egress are dependent on processing

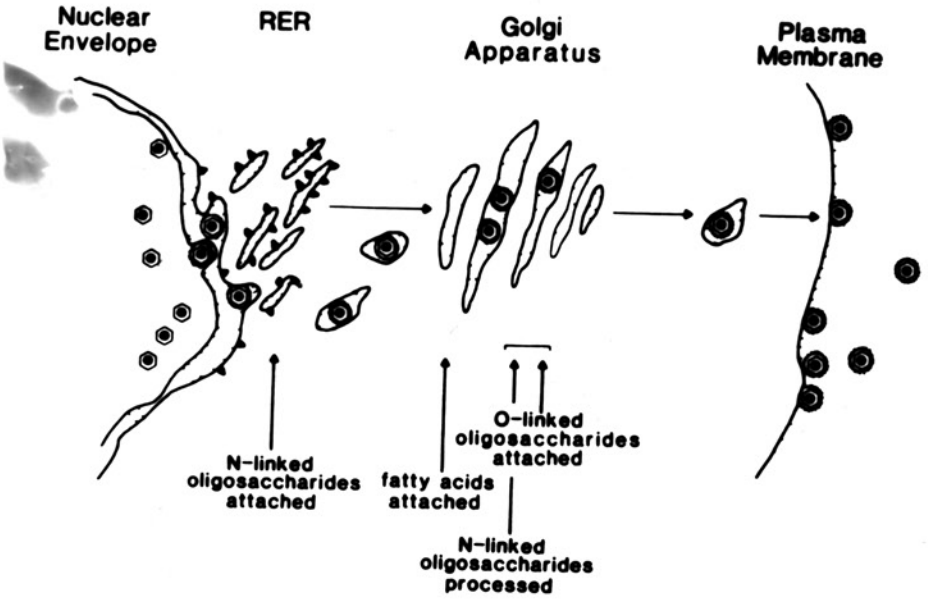


FIGURE 4

Diagram illustrating HSV envelopment and egress. This diagram shows pathways by which HSV glycoproteins are transported to the cell surface and indicates the intracellular sites at which specific events in post-translational processing of the glycoproteins occur. (Reproduced with permission from Spear, 1985)

within the Golgi (Johnson and Spear, 1982; Johnson and Spear, 1983). Infectious particles are released either by transport from the Golgi to the plasma membrane or upon cell lysis. Some fusion-inducing strains can also spread intercellularly by fusion between the plasma membranes of infected cells (reviewed in Spear, 1985).

The HSV genome

The HSV genome is a double-stranded, linear DNA molecule, approximately 150 kbp in length (Kieff *et al*, 1971; McGeogh *et al*, 1988b). The DNA is comprised of two covalently linked segments, designated long (L) and short (S). Each segment consists of unique sequences (U_L and U_S) flanked by inverted repeat sequences, terminally and internally located (i.e. TR_L and IR_L) relative to the linear structure (Sheldrick and Berthelot, 1975)(Fig.5). The L and S segments can invert relative to one another, resulting in four isomeric arrangements of the genome which arise in equimolar amounts in infected cells; one isomer is defined as the prototype arrangement (Roizman, 1979). The ability of the viral genome segments to invert relative to one another is not required for viral replication in cell culture (Jenkins and Roizman, 1986).

The HSV-1 genome demonstrates some similarity with other human herpesviruses in its structure and genetic contents (reviewed in McGeogh, 1989). In general, the genomic structures consist of linear, double-stranded DNA

molecules with 1 or 2 unique segments and the presence of terminal (and sometimes internal) direct repeat elements (Fig.5). Although the genomic size, G + C content, nucleotide sequence, and organization of repeat elements vary among these viruses, the comparison of the complete genomic sequences for EBV (Baer *et al*, 1984), VZV (Davison and Scott, 1986), HSV-1 (McGeogh *et al*, 1988b), and HCMV (Chee *et al*, 1990) reveals some similarities in sets and layouts of genes. For the alphaherpesviruses, HSV-1 and VZV, the gene layouts appear to be grossly co-linear, and most of the corresponding pairs of genes in HSV-1 and VZV show recognizable similarities in their predicted amino acid sequences (Davison and McGeogh, 1986; Davison and Scott, 1986). Comparison of EBV and VZV gene content and arrangement (which have been subsequently applied to HSV-1) indicate 29 pairs of homologous genes by amino acid sequence and 14 by genomic location, size, and hydropathy profile of the encoded proteins; however, there are several genes which are unique to each virus (Davison and Taylor, 1987, McGeogh *et al*, 1988b). Although the HCMV genomic structure is similar to HSV in its layout of repeats and unique segments, it is much larger (HSV 152 kb; HCMV, 230 kb) and contains many distinct genes which show no similarity to genes of other herpesviruses (Chee *et al*, 1990).

Identification of ORFs and encoded functions

For HSV-1, at least 72 open reading frames (ORFs),

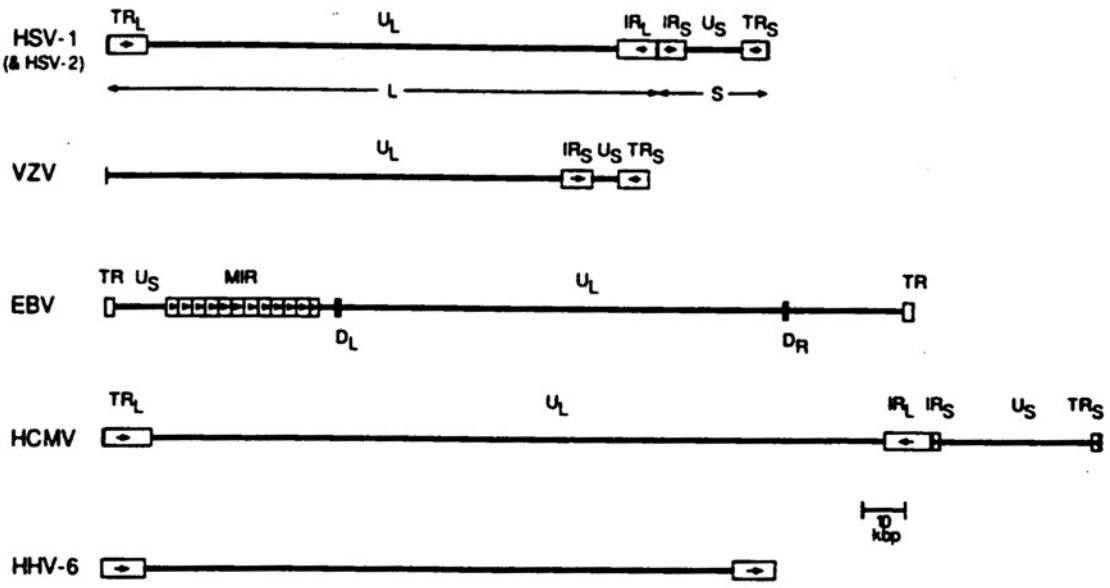


FIGURE 5

Gross structures of the genomes of the human herpesviruses. Each linear genomic DNA is shown with unique sequences as *heavy lines* and repeat elements as *boxes* with relative orientations indicated by *arrows*. U_L and U_S, long and short (L and S) unique sequences; TR and IR, terminal and internal repeat sequences; MIR, major internal repeat; D_L and D_R are each approximately 1 kb in length, and their sequences are almost identical to each other. (Reproduced with permission from McGeogh, 1989)

encoding 70 distinct proteins, have been identified based on the DNA sequence (McGeogh *et al*, 1988b)(Fig.6). Precise functions have been defined for at least 24 proteins (reviewed in McGeogh, 1989); however, the functions of the majority of HSV genes have been only partially characterized or remain unknown.

The products of HSV ORFs have been identified using a variety of genetic and biochemical techniques. In the past, many genetic studies of HSV depended upon the use of temperature-sensitive (*ts*) mutants in combination with marker rescue (cotransfection of a mutant viral genome and a wild-type DNA fragment); such mutations have been isolated in at least 22 genes. Insertion and deletion mutants have been made in a number of genes by transferring a DNA fragment containing the mutation and a selectable marker to the viral genome by homologous recombination with flanking DNA sequences.

At least 20 HSV genes have been identified as nonessential for growth in cell culture. However, it is believed that, through evolution and selective pressure placed on these viruses, all viral genes are essential in some way in viral replication or pathogenesis, and that the environmental conditions that necessitate these gene functions have yet to be identified. For example, the effects of mutations in some "nonessential" genes can be observed by varying cell culture conditions or studying

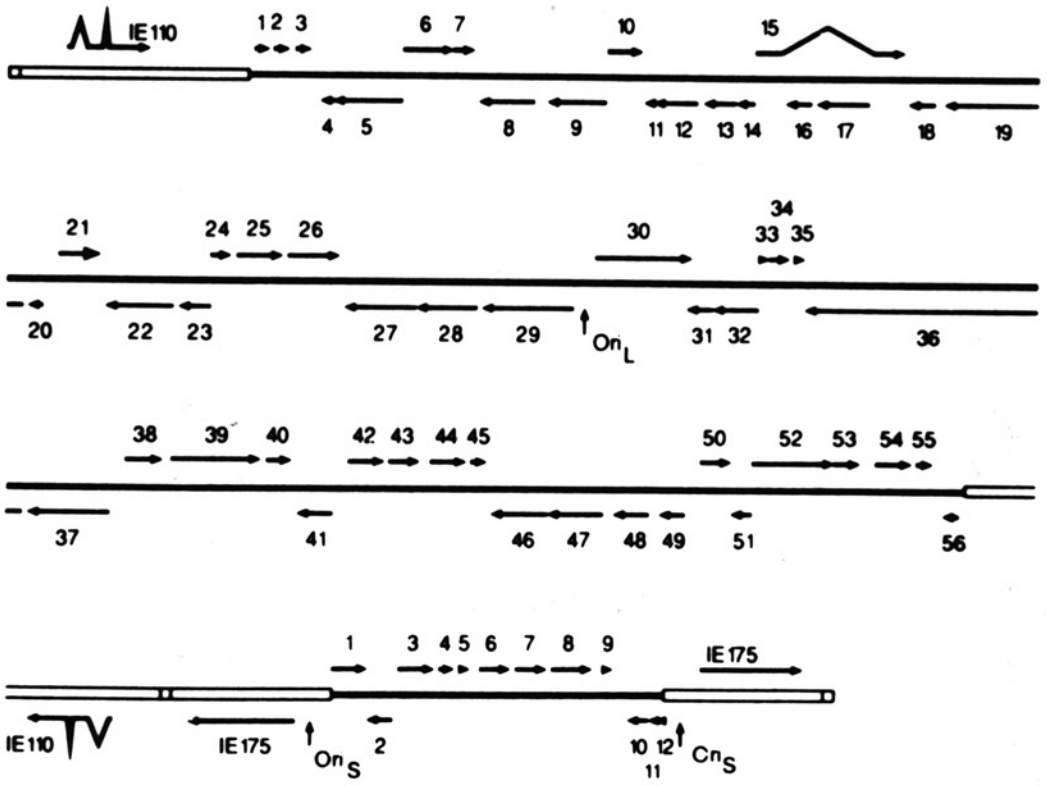


FIGURE 6

Organization of the genome of HSV-1. The linear genome of HSV-1 is represented on four successive lines of 40 kbp each. Locations of open reading frames are shown by *arrows*, with splicing within coding regions indicated. In the top three lines genes UL1-UL56 are shown as 1-56, and in the bottom line genes US1-US12 as 1-12. Locations of origins of DNA replication (ori_L and ori_S) are indicated. (Reproduced with permission from McGeogh, 1989)

pathogenesis in animals (Weller *et al*, 1988). Genes essential for HSV replication in cell culture can be mutated (creating a null mutation) and complemented in a cell line carrying the appropriate DNA fragment, creating a permissive cell line (Benjamin, 1970). Such "host-range" (*hr*) mutants have been isolated in at least 7 HSV genes. For at least 27 genes, no information regarding their essential versus nonessential nature and function exists yet.

HSV DNA binding proteins

Viral DNA binding proteins have been purified from HSV-infected cells by affinity chromatography, using DNA cellulose and agarose columns (Bayliss *et al*, 1975; Powell and Purifoy, 1976; Purifoy and Powell, 1976). Genetic and biochemical studies indicate that some of these DNA binding proteins are directly or indirectly involved in DNA replication (reviewed in Challberg and Kelly, 1989; Weller, 1991; Olivo and Challberg, 1991) or involved in transcriptional regulation (Papavassiliou *et al*, 1991; Preston *et al*, 1988). Also, there is evidence that sequence-specific DNA binding proteins are required for encapsidation of viral DNA progeny (Chou and Roizman, 1989).

Three distinct types of DNA binding protein-DNA interactions have been characterized in HSV: (1) direct binding, sequence-specific, (2) indirect binding, sequence-specific, and (3) direct binding, nonspecific (sequence-independent). The HSV UL9 protein is one example of a DNA

binding protein that binds DNA directly in a sequence-specific manner. UL9 binds a palindromic sequence located at the origin of replication (reviewed by Olivo and Challberg, 1991) and is required for DNA replication. The virion protein, αTIF (VP16, Vmw65), although unable to bind DNA itself, forms a complex with the cellular Oct-1 protein (also known as αH1, OTF-1, or NF-III). The Oct-1 protein binds DNA at a consensus sequence (the Oct-1 element) located within the HSV α gene promoters, and the indirect binding of αTIF results in transactivation of α genes (Preston *et al*, 1988; Stern *et al*, 1989; Kristie *et al*, 1989). The ICP8 (Infected Cell Protein 8) and UL42 proteins of HSV both strongly bind to DNA directly, but not at any particular sequence (Ruyechan, 1983; Ruyechan and Weir, 1984; Vaughn *et al*, 1985; Gallo *et al*, 1988). In this study, this nonspecific (sequence-independent) DNA binding activity was assessed for the UL37 protein. Our experiments led to the hypothesis that DNA binding exhibited by the UL37 protein is dependent upon its interaction with ICP8; therefore, UL37 displays an indirect and nonspecific (sequence-independent) type of interaction with DNA. The significance of this interaction can be appreciated in light of our understanding of the role of ICP8 in HSV replication.

ICP8 and its role in HSV replication

Biochemical properties

ICP8 is an abundant protein in HSV-infected cells that

has many properties characteristic of a class of replication proteins (i.e. phage T4 gene 32, adenovirus DNA binding protein, and *E.coli* single-strand binding protein [SSB]) known as helix-destabilizing proteins (also known as SSBs; Kornberg and Baker, 1992; reviewed in Chase and Williams, 1986). These proteins bind more tightly to single-stranded (SS) than double-stranded (DS) DNA, and binding to SS DNA is cooperative and independent of sequence (Purifoy and Powell, 1976; Ruyechan and Weir, 1984; Ruyechan, 1983).

ICP8 demonstrates the highest affinity for DNA among the HSV DNA binding proteins (Powell and Purifoy, 1976). ICP8 lowers the melting temperature of poly(dA)-poly(dT) duplexes (Powell *et al*, 1981), holds DNA in an extended conformation with a defined stoichiometry (protein:nucleotide ratio)(Ruyechan, 1983), and shows a small stimulatory effect on purified DNA polymerase activity on activated templates (Ruyechan and Weir, 1984). Like other helix-destabilizing proteins, ICP8 is capable of binding RNA, but with lower affinity than DNA (Ruyechan and Weir, 1984).

Role in DNA replication

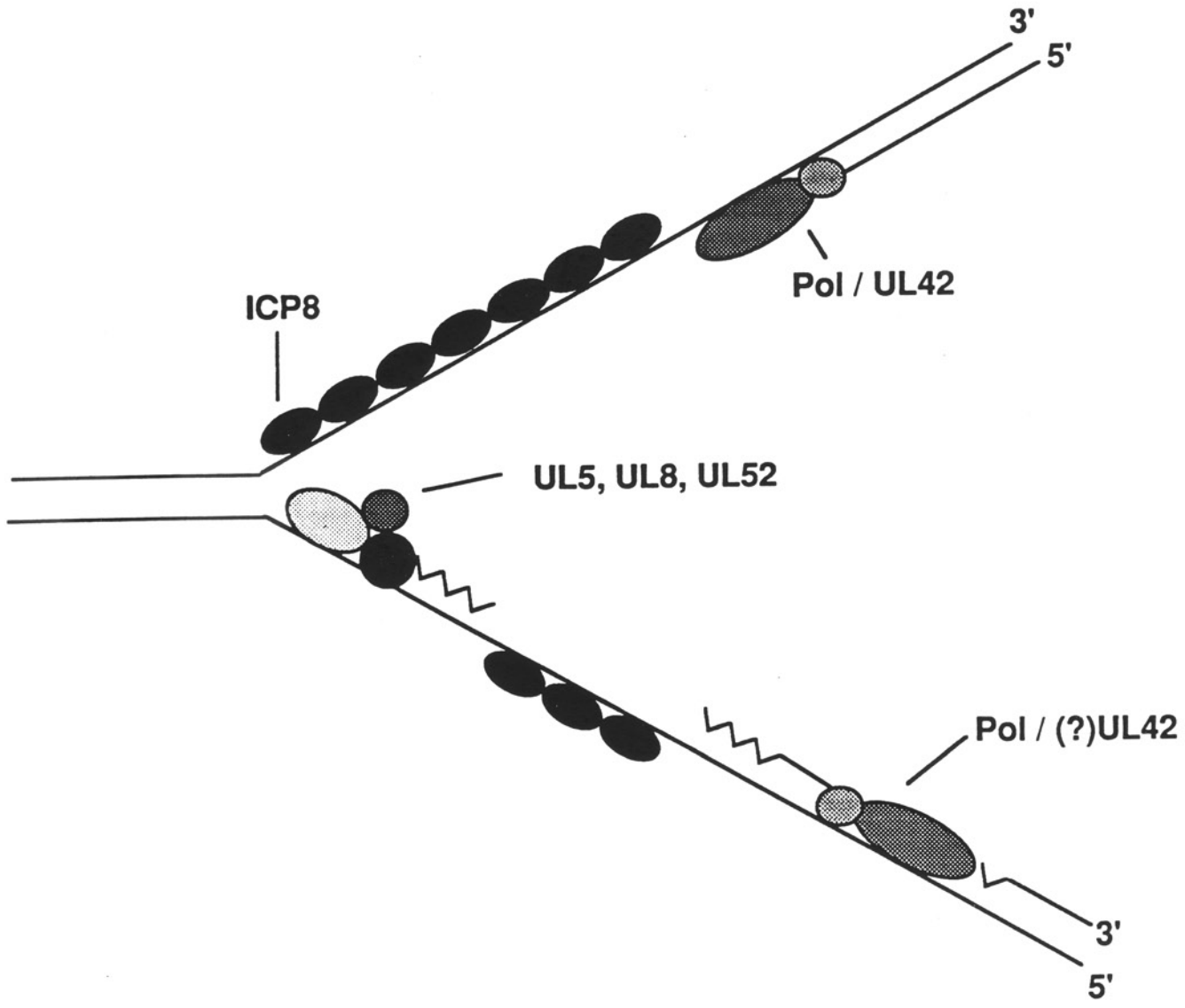
It is assumed that the function of ICP8 is analogous to other helix-destabilizing proteins--to bind to SS DNA at the replication fork formed by unwinding parental duplex DNA and to facilitate use of these strands as templates for DNA polymerase. ICP8 is essential for viral replication and DNA

synthesis as demonstrated by viral DNA(-) *ts* mutants (Conley *et al*, 1981; Weller *et al*, 1983) and has been identified as one of 7 genes necessary and sufficent for DNA replication of an HSV *ori*-containing plasmid in a transient transfection assay (Wu *et al*, 1988). While the overall mechanism of HSV DNA replication is not competely understood, a model of the initiation of HSV DNA replication has been deduced from this replication system by taking into account the biochemical characteristics of each HSV protein involved and comparisons with other better characterized replications systems (i.e. *E.coli oriC*, λ, and SV40)(reviewed in Olivo and Challberg, 1991)(Fig.7). The origin-binding protein (UL9) initiates ATP-dependent local unwinding of the duplex DNA, allowing for the entry of a helicase/primase complex (UL5, UL8, and UL52) which enlarges the unwound region and primes nascent strands at the replication fork. Elongation of both leading and lagging strands is most likely facilitated by a DNA polymerase (Pol) and UL42 complex. It has been suggested that the UL42 DNA binding protein serves as a "tether" for DNAPol to its template, resulting in processivity. The ICP8 protein binds to the single-stranded regions of the replication fork, thus stabilizing the multi-protein/DNA structure. Two enzymatic functions presumably required for DNA replication include a topoisomerase and ligase activity; if required, such functions may be supplied by the host cell.

The identification of the 7 HSV genes required directly

FIGURE 7

A schematic summary of the biochemical activities of the HSV-encoded replication proteins. This diagram includes six of the seven replication proteins identified by the transient transfection system described by Wu *et al*, 1988. The proposed model for DNA replication depicted in this figure is described in the text. (Reproduced with permission from Olivo and Challberg, 1991)


3'
5'
Pol / UL42
ICP8
UL5, UL8, UL52
Pol / (?)UL42
3'
5'

for DNA replication in the transient transfection assay described above corresponds with the findings of genetic studies, showing that viral mutants in each of these 7 genes result in DNA(-) phenotypes in cell culture (reviewed in Weller, 1991). Mutation of other HSV-encoded DNA replication functions, identified by enzymatic assays of infected cell extracts, demonstrate a DNA(+) phenotype in cell culture (reviewed in Weller, 1991). These include enzymes involved in both nucleotide metabolism, such as ribonucleotide reductase (RR, UL39 and UL40), thymidine kinase (tk, UL23), and dUTPase (UL50), and DNA repair, such as uracil-DNA glycosylase (UL2). These genes are nonessential for virus replication in cell culture, but may be required for DNA synthesis in growth-arrested cells *in vivo* such as the neuron.

Several *ts* mutants which make viral DNA, but are defective in processing it into capsids, have been isolated including the alkaline exonuclease (DNase, UL12) gene and several genes whose functions are unknown (reviewed in Weller, 1991). The precise role of alkaline exonuclease in viral DNA replication and processing has not been clearly established. Recently, Thomas *et al* (1992) have proposed that the alkaline exonuclease may be involved not only in packaging viral DNA, but also in DNA replication complexes early in infection since some studies show an association between the alkaline exonuclease and both ICP8 and DNAPol.

Nuclear localization

After its synthesis in the cytoplasm, ICP8 is transported to the nucleus (Fenwick *et al*, 1978). The intranuclear distribution of ICP8 differs in the presence and absence of DNA synthesis (Knipe and Spang, 1982). Immunofluorescence studies using an ICP8 monoclonal antibody indicate that the ICP8 associates with the nuclear matrix at "prereplicative sites" prior to the onset of DNA synthesis, and patterns of nuclear compartmentalization change with the onset of DNA replication (Quinlan *et al*, 1984). In addition, ICP8 facilitates the redistribution of cellular DNA replication complexes to these viral prereplicative sites (de Bruyn Kops and Knipe, 1988). It has been proposed that ICP8 is responsible for organizing nuclear structures for the HSV replication complexes (de Bruyn Kops and Knipe, 1988).

Genetic studies have demonstrated that the C-terminal 28 residues of the ICP8 protein are sufficient for nuclear localization (Gao and Knipe, 1989). Nuclear localization signals (NLSs) have been identified in other nuclear proteins as short stretches of basic amino acids (i.e. SV40 T Ag, $P^{126}KK^{128}KRKV^{132}$; Kalderon *et al*, 1984). Currently, two distinct types of NLSs have been identified: a single short basic tract resembling the NLS of SV40 T Ag, or bipartite signals like those of Xenopus nucleoplasmin or steroid receptors consisting of discontinuous clusters of basic

residues (Robbins *et al*, 1991). By comparison with other reported NLSs, the ICP8 amino acid sequence indicates a bipartite signal at the C-terminus, RKR-X_{11}-PDKK.

Such NLS regions are believed to exhibit a secondary structure which is recognized by nuclear pore complexes, facilitating the unidirectional, selective entry of proteins into the nucleus (Yoneda, 1988; Yamasaki, 1989; reviewed in Dingwall and Laskey, 1986; Roberts, 1989). Knipe and Smith (1986) reported that infection with an ICP4 *ts* mutant, whose ICP4 protein fails to be efficiently transported to the nucleus at the nonpermissive temperature, impairs the nuclear transport of ICP8 and ICP0, but not ICP27. This suggests that the mutant ICP4 protein may block the nuclear uptake of other proteins by blocking the nuclear translocation machinery. Also, there may be different classes of nuclear pores.

In this study, the nuclear localizing potential of ICP8 was utilized to further investigate the association of ICP8 and the UL37 protein of HSV demonstrated by DNA binding experiments.

Functional domains of ICP8

Genetic evidence for functional domains of ICP8 is based on (1) *ts* mutants (Lee and Knipe, 1983; Leinbach *et al*, 1984; Quinlan *et al*, 1984; Ruyechan *et al*, 1986; Gao *et al*, 1988), (2) expression of cloned wild-type, mutant, and truncated versions of the ICP8 gene by transient transfec-

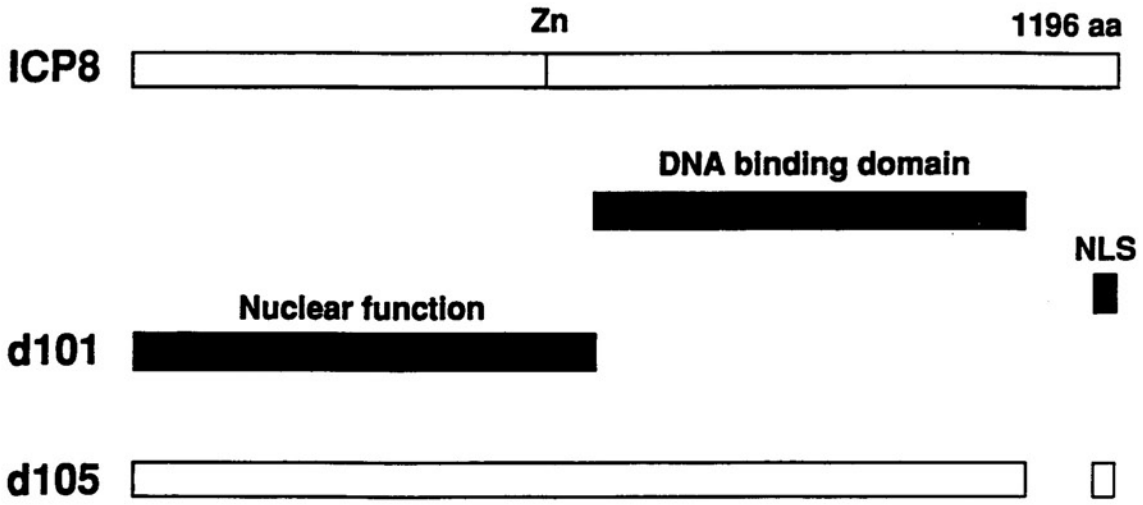
tion or *in vitro* transcription and translation, and analysis of tryptic fragments of ICP8 (Gao *et al*, 1988; Leinbach and Heath, 1988; Leinbach and Heath, 1989; Wang and Hall, 1990), and (3) *hr* mutants in permissive cells lines (Gao and Knipe, 1989). Taken together, the findings are represented in Fig.8 and are summarized here. The C-terminus (aa 564-1160) is sufficient for DNA binding. The N-terminus is not absolutely required for SS DNA binding, but may be involved in stabilizing the binding activity of the C-terminus; this functional domain exists separately from DNA binding and nuclear localization, yet is important for viral growth and DNA replication. A zinc-binding domain (aa 499-512), which is common to many DNA binding proteins, can be disrupted by substituting glycine for each cysteine in the zinc finger, and the protein retains some ability to bind DNA. The C-terminal 28 residues are sufficient for nuclear localization. In addition, an interaction between ICP8 and DNAPol exists since 5 ICP8 *ts* mutants exhibit altered sensitivity to inhibitors of DNA synthesis (Chiou *et al*, 1985).

Role of ICP8 in transcriptional regulation

ICP8 is required not only for viral DNA replication, but also for normal regulation of viral gene expression. A variety of ICP8 mutants show altered expression of α, β, $\gamma 1$, and $\gamma 2$ mRNAs (Conley *et al*, 1981; Godowski and Knipe, 1983; Godowski and Knipe, 1985; Godowski and Knipe, 1986; Orberg

FIGURE 8

Functional domains of ICP8. Analysis of a variety of ICP8 mutants indicates that the DNA-binding region of ICP8 is located within residues 564-1081 and that the C-terminal 28 residues of ICP8 can function as a nuclear localization signal. Mutant d101 ICP8 which is missing residues 17-563 localizes to the nucleus and binds to SS DNA but fails to promote viral DNA replication (Gao and Knipe, 1989); this indicates that the N-terminal half of ICP8 has a nuclear function other than DNA binding. The d105 mutant ICP8 has a deletion of 1082-1169 and exhibits a *trans*-dominant negative phenotype (Gao and Knipe, 1991).


Zn
1196 aa
ICP8
DNA binding domain
NLS
Nuclear function
d101
d105

and Schaffer, 1987). However, no common effect, either positive or negative regulation of the different temporal classes, has been consistently reported. Recently, Gao and Knipe (1991) reported creation of a cell line expressing a deletion mutant ICP8 protein (d105, Fig. 8) which has a *trans*-dominant effect on γ gene expression during HSV infection. Their findings strongly suggest that ICP8 may play a role late in infection in stimulating γ gene expression. They propose that late in infection ICP8 may optimize transcription by binding single-stranded regions of progeny DNA, keeping promoter regions open for transcription, and that ICP8 may require interactions with other proteins to facilitate this modified ICP8 function and potentially a specific recognition of late gene promoters.

Specific aims

To enhance our understanding of the complex process of replication of HSV and ultimately its pathogenesis, elucidation of the functions encoded by its genome is highly desired. Unfortunately, few of the HSV gene functions are precisely defined; many are only partially understood or remain unknown. The function of the UL37 gene is completely unknown, and its gene product was chosen as a focus for study.

The overall aims of this dissertation were to clone and express the UL37 gene in order to produce UL37-specific immunological reagents, to identify and characterize the

UL37 protein in HSV-infected cells and virions, and to investigate, by DNA binding and nuclear localization studies, some interesting biochemical features of the UL37 protein including a newly identified association with the ICP8 protein.

MATERIALS AND METHODS

Cells and virus propagation

Cell culture

CV-1 and Vero cells (American Type Culture Collection) and U-47 cells (obtained from Dr. Priscilla Schaffer, Harvard Medical School, Dana-Farber Cancer Institute, Department of Microbiology and Molecular Genetics) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) Serum Plus (SP, Hazelton) and 50 μg/ml gentamicin (U.S. Biochemicals, Inc.), approximately 40 ml/flask, in 150 cm^2 flasks (Costar) or 32 oz glass flasks (Owens-Brockway) and incubated at 37°C with approximately 5% CO_2. When cell monolayers reached confluency, they were washed with 5-10 ml phosphate buffered saline (PBS), trypsinized with 2-5 ml trypsin/EDTA (0.25% in buffered saline/0.02% [v/v], JRH Biosciences), resuspended in growth medium, and seeded into new flasks.

Viral stocks

Herpes simplex virus type 1 (HSV-1) and vaccinia viruses were grown in either CV-1 or Vero cells. The wild-type strains of HSV and vaccinia virus used in this study were HSV-1(F), HSV-1(17), and vaccinia (WR). The properties of HSV-1(F) and vaccinia (WR) have been previously described (Ejercito et al, 1968; Chakrabarti *et al*, 1985). Viral stocks were prepared by infecting 150 cm^2 flasks of

confluent cells at a multiplicity of infection (MOI) of 0.001 to 0.01 pfu/cell. For viral infections, virus was diluted in EMEM and allowed to adsorb to the cells for 1 hr at room temperature with rocking. Unadsorbed virus and medium were then aspirated and replaced with growth medium, 40 ml/flask. Infected cells were incubated at 37°C and approximately 5% CO_2 until 90 to 100% cytopathic effect (CPE) was observed (generally two to three days). Infected cells were scraped and collected in 50 ml conical tubes (Corning). Following centrifugation at 1000 rpm for 2-5 minutes in an IEC (model HN-SII) benchtop centrifuge, the infected cell pellets were resuspended in growth medium at 1 ml/flask. Intracellular virus was released, either by three cycles of freezing and thawing or by a brief 15-30 s sonication. The viral suspensions were stored as 1-2 ml aliquots in Wheaton vials at -70°C. Occasionally, an equal volume of pasteurized milk (nonfat powdered milk prepared as directed on package and autoclaved for 15 min, three times, consecutively) was added to stabilize the virus infectivity during prolonged storage.

Viral plaque assay

Virus titers were determined by plaque assay. Serial dilutions of virus were made in EMEM, typically 10^{-1} to 10^{-8}, each in a total volume of 1 ml. Confluent CV-1 cells grown in 6-well cell culture plates were infected by aspirating the growth medium and adding the diluted virus. Using the

10^{-8} to 10^{-3} dilutions, 0.5 ml aliquots were added to each well, in duplicate, and the virus was allowed to adsorb for 1 hr, rocking at room temperature. Unadsorbed virus and medium were aspirated and replaced with a methylcellulose overlay (EMEM-5%SP with 1% [w/v] methylcellulose) at 3 ml/well. Infected cells were incubated at 37°C and approximately 5% CO_2 until clearly visible plaques were observed, typically 2-3 days, and then carefully washed with PBS and stained with 0.1% (w/v) crystal violet in 50% (v/v) methanol for 20 minutes, washed in water, and air-dried. Plaques were counted on a dissecting scope (Bausch and Lomb Stereozoom 7) and viral titers determined.

Recombinant viruses

The HSV-1 recombinant, d21, was obtained from Dr. Priscilla Schaffer. Virus propagation, viral stock preparation, and plaque assay for virus titers were performed in an identical fashion to that of HSV-1 except d21 was propagated on U-47 cells (Orberg and Schaffer, 1987).

The vaccinia recombinant, V37, was isolated by Dr. Michael Pensiero and was derived by transfection of pJF34 (described in Results, Section I) into vaccinia-infected cells. V37 was propagated on CV-1 cells, and viral stocks were prepared as previously described. V37 plaque assays were also performed as previously described with the exception that the overlay contained 300 μg/ml X-gal and 1%

(w/v) low gel temperature agarose instead of methyl-cellulose. The agarose overlay was maintained at 37°C prior to layering on infected cells. Unadsorbed virus and medium were aspirated, and agarose overlay layered on top of infected cells at 3 ml/well and allowed to gel at room temperature for 30 minutes prior to incubation of cells. Blue plaques were visualized 3-5 days postinfection and counted to determine virus titer.

An HSV-1 recombinant, hrR3, an ICP6 mutant containing an inserted ICP6::*lac*Z cassette, obtained from Dr. Sandra Weller (University of Connecticut Health Center, Department of Microbiology; Goldstein and Weller, 1988a) and potential UL37 recombinants were screened by plaque assay in CV-1 cells and CV37 cells (see Results, Section IV), respectively, as described for HSV-1 except an additional X-gal overlay (1-2 ml/well) was layered on top of the pre-existing methylcellulose following visualization of plaque formation. The second overlay consisted of PBS and 300 μg/ml X-gal. Blue plaques developed within 8-24 hr following overlay.

Generation of cell lines & isolation of viral recombinants

DNA transfection

DNA transfections of plasmid molecules into Vero or CV-1 cells were performed using either the $CaPO_4$ method as described by Graham and Van der Eb (1973) or liposome-mediated transfection (Lipofectin, BRL). For these

experiments, 0.5-10 μg of plasmid DNA was used to transfect 60 mm plates containing 80% confluent CV-1 or Vero cells. Viral DNA transfections were performed using the $CaPO_4$ method.

Isolation & screening of cell lines

Vero and CV-1 cells co-transfected with G418 resistance and UL37 encoding plasmids were grown to confluency, trypsinized, and seeded into 100 mm plates containing growth medium plus G418 at 400 μg/ml (Geneticin, Gibco) for the selection of G418 resistant cells. G418 resistant foci were picked and seeded into 24-well plates. Once confluent, each cell line was subsequently grown to greater numbers until finally reaching confluency in 150 cm^2 flasks. G418 resistant cells were maintained in growth medium plus G418 at 200 μg/ml and, following several passages, G418 supplementation was used for every fifth passage.

High molecular weight cellular DNA was prepared from each cell line following trypsinization by resuspending the cell pellet in TNE (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 10 mM EDTA) with 0.2% (w/v) SDS and 1 mg/ml proteinase K. The lysate was incubated at 37°C overnight and then deproteinized by phenol/chloroform extraction followed by chloroform extraction. The final aqueous phase was then dialyzed against 2 L TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) overnight at 4°C. DNA concentrations were determined by the diphenylamine technique (Burton, 1956), using varying

amounts of deoxyadenosine to generate a standard curve.

High molecular weight DNA prepared from each cell line was screened by either a dot blot assay or polymerase chain reaction (PCR) analysis. For dot blot analysis, the DNA was denatured with 1.5M NaCl and 0.5M NaOH for 10 min and then neutralized with 3M Na acetate (pH 4.8) for 10 min. The DNA samples were then transferred to a Biodyne membrane by use of a dot blot apparatus, and then screened by DNA hybridization (see Southern blots) using a plasmid or DNA fragment as probe. For detection of UL37 DNA sequences by PCR analysis, UL37 specific primers were selected using the primer detection program, Primer Detective (Clonetech). Oligonucleotides were synthesized by M.N. Flora (USUHS). Each PCR reaction consisted of at least 20 ng cellular DNA, 1X Taq I buffer, 1 μg of each primer, 300 μM each deoxynucleotide triphosphate (dNTP), 2.5 U Taq polymerase (Promega), and deionized distilled H_2O in a total volume of 100 μl. The reaction mix was boiled for 10 minutes to denature the DNA and placed on ice prior to the addition of Taq polymerase. PCR reactions were performed in a thermocycler (Perkins-Elmer-Cetus) by denaturing at 96°C for 2 min, annealing at 55°C for 1.5 min, and extending at 72°C for 2 min for a total of 30 cycles. A 10 μl aliquot of each PCR sample was analyzed by agarose gel electrophoresis.

Purification of viral DNA

CV-1 cells were infected with HSV-1(F) or HSV-1(strain

17) at an MOI of 1. Once CPE developed in 90-100% of the cells, the cells were scraped, pelleted, and resuspended in 10 ml TE. The viral DNA was purified by equilibrium density centrifugation in NaI gradients essentially as described by Post *et al* (1980). To release cytoplasmic viral DNA, the cells were placed on ice, lysed with 10% (v/v) Nonidet P-40 (NP40), and the nuclei pelleted by centrifugation in a Beckman SS34 rotor at 5,000 rpm for 5 min at 4°C. The cytoplasmic extract was deproteinized by treatment with EDTA and sodium dodecyl sulphate (SDS) at a final concentration of 25 mM and 0.5% (w/v), respectively, and addition of 100 µl proteinase K (10 mg/ml). The lysate was incubated at 37°C overnight, ethidium bromide added, and the viral DNA isolated on a NaI gradient following centrifugation in a Beckman VTi50 rotor for 2 days at 45,000 rpm at 20°C. The viral DNA band was visualized by use of a hand-held UV light and harvested using a large gauge needle. Ethidium bromide was removed by butanol extraction and the NaI removed by dialysis against TE overnight. Following dialysis, the viral DNA was collected and stored at 4°C. Infectivity of each viral DNA preparation was assessed by $CaPO_4$ transfection on CV-1 cells.

Viral recombinants

Viral recombinants were generated by $CaPO_4$-mediated co-transfection of infectious viral DNA and plasmid DNA. For each experiment, varying amounts of plasmid DNA (0.5-2.0 µg)

were used. Once 90-100% CPE had developed, the cells from each 60 mm dish were harvested and frozen and thawed three times to release intracellular virus. Plaque assays using the methylcellulose overlay were performed with each virus mixture for determination of virus titer and to screen for β-galactosidase$^+$ plaques using the secondary X-gal overlay described earlier. Blue plaques were picked and re-plaqued on confluent CV-1 cells in 100 mm dishes several times for purification and with X-gal screening repeated to confirm or rule out the β-galactosidase$^+$ phenotype.

Gel electrophoresis

Agarose gel electrophoresis

To separate DNA fragments, usually greater than 0.5 kb in size, 0.8% (w/v) agarose slab gels were used. Gels were prepared by adding 2.4 g of agarose powder to 300 ml of 1X TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). The agarose was dissolved by boiling in a microwave oven, poured into casting trays (BRL) with tape-sealed ends, and the desired comb inserted. The size of the casting tray and comb selected varied depending on the size of the gel desired for adequate separation of DNA fragments, the number of samples, and volume of samples. Once hardened, the gel and casting tray were submerged in 1X TBE running buffer in an electrophoresis rig (either BRL Horizon 58, Model H6, H5, or H4), the comb carefully removed, and samples loaded into the wells. A 10X loading buffer consisting of 0.25% (w/v)

bromophenol blue, 0.25% (w/v) xylene cyanol, and 25% (w/v) ficoll was added to each sample prior to loading. At least one well per gel contained a mixture of DNA fragments of known size, typically a 1 kb ladder (Gibco), to approximate the size of the DNA fragments in each sample. Samples were electrophoresed at constant voltage, the current not exceeding 50 mA. DNA fragments were visualized by ethidium bromide staining and use of a UV light. The gel was immersed in an 50 μg/ml ethidium bromide solution (either in H_2O or 1X TBE) for 10 to 20 minutes and then destained in H_2O for at least 10 minutes prior to visualization of bands on a UV light box, emitting at 254 nm, and photography using Polaroid type 55 or 70 film with a Polaroid MP-4 camera. If DNA fragments were to be cut out from the gel and eluted for cloning, visualization of bands was achieved by use of a hand-held long wave UV light, minimizing damage to the DNA, and the gel was photographed after the band(s) were excised from the gel.

To assess polymerase chain reaction (PCR) products, whose sizes were less than 500 bp in length, 2% (w/v) agarose gels in 1X TBE were used. The gels were run, stained, and photographed as described above.

Formaldehyde/agarose gel electrophoresis

Deproteinized RNA from *in vitro* transcription reactions were assessed by vertical formaldehyde/agarose gel electrophoresis. A vertical gel rig (SE 600 series, Hoefer

Scientific Instruments [HSI]) and 3 mm spacers were used. After 3 g agarose was dissolved in 144 ml double distilled H_2O by boiling in a microwave oven, 20 ml of 10X MOPS buffer (0.2M 3-[N-morpholino] propanesulfonic acid [MOPS] pH 7.0, 50 mM sodium acetate, 10 mM EDTA) and 35 ml of 37% formaldehyde were added, the gel poured, and comb inserted. Following cooling of the gel, the screws were slightly loosened in order to carefully remove the comb, and the gel was inserted between upper and lower buffer chambers. Buffer chambers were filled with 1X MOPS buffer and RNA samples prepared (see *in vitro* transcription, Materials and Methods) and loaded. The electrophoresis was carried out at 100 V, constant voltage, for 2-3 hours. Following electrophoresis, the gel was removed, wrapped in cellophane wrap (while wet), and autoradiographed on Kodak XAR-5 film.

Polyacrylamide gel electrophoresis

Proteins were separated using denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on a procedure developed by Laemmli (1970). Large gels, 16 cm X 16 cm or 16 cm X 20 cm, were run on Bio-Rad Protean II vertical gel rigs, and minigels were run on Bio-Rad Mini-Protean II vertical gel rigs, both using 1.5 mm spacers and combs.

Gels were made from a 30% (w/v) acrylamide stock containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide (bisacrylamide) in distilled H_2O.

Lower gel buffer (LGB) consisted of 0.1% (w/v) SDS in 375 mM Tris-HCl (pH 8.8). Upper gel buffer (UGB) consisted of 0.1% (w/v) SDS in 125 mM Tris-HCl (pH 6.8). The 1X running buffer consisted of 25 mM Tris, 192 mM glycine, and 3.5 mM SDS. A stock solution of 10% (w/v) ammonium persulfate (APS) in H_2O was used along with N,N,N',N'-tetramethyl-ethylenediamine (TEMED) to facilitate crosslinking of the acrylamide.

For large gels, a 2-5 mm acrylamide plug was poured at the bottom of each gel to prevent leaking. The plugs were prepared by combining 5 ml 30% acrylamide stock, 1.75 ml 4X LGB, 25 μl APS, and 25 μl TEMED. The separating gels containing 9% (w/v) acrylamide in LGB with 3.6 μl/ml APS and 0.48 μl/ml TEMED were poured and overlaid with 2 mm of 0.1% (v/v) H_2O-saturated N-butanol to create an even interface between separating and stacker gels. When polymerization was complete, the butanol was rinsed off with deionized H_2O and replaced with 1X LGB if gels were to be stored at room temperature prior to use (usually overnight) or a stacking gel was added for immediate use. The stacking gels consisting of 4.5% (w/v) acrylamide in UGB with 3 μl/ml APS and 1.6 μl/ml TEMED were poured, combs inserted, and allowed to polymerize for 15-30 minutes. Wells were carefully rinsed with deionized H_2O and gels inserted into electrophoresis rigs. The upper buffer chamber was filled with fresh 1X running buffer and the tank filled at least

half way with fresh or used 1X running buffer for large gel rigs and completely for minigel rigs to prevent leakage.

Protein samples in 1X sample buffer (2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 50 mM Tris-HCl [pH 6.8], 4% [v/v] glycerol, and 0.15 mM bromophenol blue) were boiled 2 minutes prior to loading. For minigels, the sample volume loaded was 20-30 μl/well. For large gels, the sample volume loaded was 50 μl/well. At least one well per gel contained a prestained high molecular weight standard (BRL). The 16 cm X 16 cm large gels were run at 120 mA-hr/gel and the 16 cm X 20 cm large gels were run at 160 mA-hr/gel, constant current, until the dye front ran into the plug, either overnight without cooling or in 6-8 hr with connection to a water-cooling apparatus (Lauda RM6). Minigels were run at 200 V until the dye front reached the end of the gels. Gels were either Coomassie or silver stained or transferred to nitrocellulose for immunoblotting.

Staining SDS-PAGE gels for proteins

Coomassie staining (Wilson, 1983) for visualization of separated proteins was achieved by rocking the gel(s) 30-60 min at room temperature in an excess of Coomassie stain/fixative consisting of 50% (v/v) methanol, 10% (v/v) acetic acid, and 0.1% (w/v) Coomassie blue in distilled H_2O, and then followed by destaining in 5% (v/v) methanol and 7% (v/v) acetic acid in distilled H_2O, for at least 3 hr, until bands were clearly visualized.

For more sensitive detection of protein bands, SDS-PAGE gels were silver stained essentially as described by Oakley *et al* (1980). The gels were soaked overnight in 50% (v/v) methanol in double distilled H_20. Silver stain was freshly prepared for each use as follows. In a small beaker, 1.4 ml concentrated NH_4OH (15 M) was added to 21 ml 0.1 M NaOH. Prepared separately, 0.8 g $AgNO_3$ was thoroughly dissolved in 4 ml double distilled H_2O and then added dropwise to the beaker. Once all the silver was dissolved, another 75 ml double distilled H_2O was added to complete the volume to 100 ml. The gels were soaked in this silver stain for 20 min, and then the stain was carefully poured off. The gels were then soaked in double distilled H_2O for 5-15 min. After decanting, a developer solution consisting of 250 μl 37% formaldehyde and 2.5 ml 1% (w/v) citric acid in a total volume of 500 ml in double distilled H_2O (mixed in this order) was added to the gels to allow for development of stained bands. After development, a fixative consisting of 10% (v/v) methanol and 10% (v/v) acetic acid in double distilled H_2O was added for 10-20 min.

Both Coomassie and silver stained gels were usually transferred to Whatmann 3MM filter paper and dried for 2 hr on a slab gel drier (Model SE 540, HSI). Alternatively, gels were soaked in a 10% (v/v) glycerol in H_2O solution, then sandwiched between two sheets of drying film (Promega), and placed in a drying frame until dry.

Immunoblots

Proteins separated on SDS-PAGE gels were transferred to 0.45 μm nitrocellulose (Schleicher & Schuell) for immunoblot analysis using either a TE50 Transphor apparatus (Hoefer Scientific) or a Bio-Rad semi-dry blotting apparatus for large gels, and a Bio-Rad Mini-transblot II for minigels. Prior to transfer, gels were soaked 15-30 min in the transfer buffer WB-1, containing 25 mM Tris, 219 mM glycine, and 20% [v/v] methanol in distilled H_2O. The gels were sandwiched, next to pre-wetted nitrocellulose, between WB-1 saturated Whatman 3MM filter paper (one on each side for transblot apparatus or two on each side for semi-dry blotting) and Scotch-brite pads for assembly in transblot cassettes. Semi-dry transfer of large gels was achieved by running at 20 V, limiting current to 0.77 A (0.3 A X area of gel [in cm^2]), for 1 hr. Transphor and transblot rigs were filled with WB-1 and, for large gels, run at 0.3 A overnight and, for mini-gels, run at 100 V for 1 hr with ice-pack cooling or 30 V overnight. Transfer efficiency was assessed by observation of the complete transfer of the set of prestained marker proteins. Following transfer, the nitrocellulose membranes were treated with 10% (w/v) nonfat milk in the binding buffer WB-2, (4 mM EDTA, 10 mM Tris-HCl [pH 7.6], 0.15 M NaCl, 0.05% (v/v) Tween 20, and 0.02% (w/v) Na azide), at 4°C at least overnight or stored in this buffer until probed with antibody.

Typically, blots were probed by sealing in a plastic bag with rabbit antiserum diluted 1:50 in 1% (w/v) nonfat milk in WB-2, and incubated at room temperature for 1-3 hr with rocking followed by extensive washing in WB-2. The bound antibodies were then detected by sealing the blots in a plastic bag with WB-2 and 1 μl/ml ^{125}I-labelled protein A (4.85 μCi/μg, NEN Research Products), incubating at room temperature for 0.5-1 hr with rocking, followed by washing again with WB-2 to remove unbound protein A. The blots were air dried, wrapped in cellophane, and autoradiographed on Kodak XAR-5 film with an intensifying screen. Densitometric scans were performed on multiple exposures of the autoradiograms with a Shimatzu laser densitometer. Alternatively, blots were put down on phosphorscreens and scanned using the Phosphorimager system (Molecular Dynamics).

Blots containing ^{35}S-methionine-labelled proteins were probed in a similar fashion as described above, and bound antibodies were detected either by use of a biotinylated secondary antibody in conjunction with avidin and biotinylated horseradish peroxidase (Vectastain ABC system, Vector laboratories) or by use of an alkaline phosphatase-conjugated secondary antibody. In either case, PBS was substituted for all washes and for dilution of secondary antibodies since Na azide contained in the WB-2 interferes with the substrate-enzyme reactions. Following incubation

with primary antibody and PBS washing, blots were incubated with secondary antibody in sealed plastic bags for 0.5-1 hr, rocking at room temperature, and then washed extensively in PBS. For Vectastain western blots, blots were additionally incubated in sealed plastic bags with ABC reagent (prepared according to the manufacturer's protocol) for 1 hr, rocking at room temperature, and washed again with PBS prior to addition of substrate.

The substrate for the horseradish peroxidase was prepared by dissolving 10 mg chloronaphthol in 1 ml ethanol and adding this dropwise to 50 ml PBS, prewarmed to 37°C, and finally adding 15 μl 30% H_2O_2. The substrate for the alkaline phosphatase was prepared by adding 44 μl NBT (nitro blue tetrazolium, 75 mg/ml in dimethylformamide) and 33 μl BCIP (bromochloroindolyl phosphate, 50 mg/ml in dimethylformamide) to 10 ml alkaline phosphatase buffer containing 100 mM Tris (pH9.5), 5 mM $MgCl_2$, and 100 mM NaCl in double distilled H_2O. Substrate was added to the blots, and bands appeared within 10-20 min. The blots were then washed with PBS, air dried, wrapped in cellophane, and wrapped in aluminum foil to eliminate exposure to light.

Plasmids

Growth and purification

Plasmid-containing bacteria were streaked for isolation on bacterial plates (LB plus agar) supplemented with the appropriate antibiotic (usually Ampicillin [Ap] since most

of the vectors contained an ampicillin resistance gene) and incubated overnight at 37°C in a non-CO_2 incubator. A single colony was picked to inoculate an overnight culture of 3-5 ml LB and incubated at 37°C with shaking at 200 rpm. Overnight cultures were used for small scale plasmid preparations, to prepare a glycerol stock, or to inoculate large scale bacterial cultures, 500 ml to 1 L LB supplemented with the appropriate antibiotic, for large scale plasmid preparations. A glycerol stock of each bacterial strain was prepared by adding 600 μl of 80% (v/v) glycerol to 400 μl of overnight bacterial culture and stored at -70°C in Wheaton vials.

Small scale plasmid preparations were performed by a boiling-lysozyme procedure. Briefly, 1.5 ml of each bacterial culture was pelleted in eppendorf tubes. The bacterial pellets were lysed by resuspending in 400 μl STET/lysozyme buffer (8% [w/v] sucrose, 5% [v/v] Triton X-100, 50 mM Tris-HCl [pH8.0], 50 mM EDTA, lysozyme 10 mg/ml) by vortexing 15 s. Bacterial lysates were boiled for 55-60 s and then spun in a Beckman microfuge at 12,000 X g at 4°C for 15 minutes. Pellets of insoluble cell components were removed with a toothpick, and plasmid DNA was precipitated by addition of 400 μl isopropranol/tube, incubation at -20°C for 5 min, and centrifugation in a microfuge at 4°C for 5 min. Isopropanol was aspirated and DNA pellets allowed to air dry. DNA pellets were resuspended in 50-100 μl TE and

stored at -20°C.

Large scale plasmid preparations were performed by an alkaline lysis followed by CsCl purification essentially as described by Maniatis *et al* (1982) except ethidium bromide was extracted by ethanol precipitation. Following phenol/chloroform extraction and ethanol precipitation, the DNA pellet was dried under vacuum, and resuspended in 50-100 μl TE or double distilled H_2O and stored at -20°C. The amount of DNA was then quantitated by measuring the absorbance at 260 nm. Plasmid identity was confirmed by restriction enzyme digestion of 0.5 μg plasmid DNA with appropriate restriction enzymes and agarose gel electrophoresis.

Vectors & Constructs

Plasmid vectors Bluescript SK+ (Stratagene) and pUC19 (BRL) were purchased from the manufacturers. The plasmid pBR329 was donated by Dr. Anthony Maurelli (USUHS). The plasmid pRB210 containing the HindIII K fragment of HSV-1(F), coordinates 0.527-0.592 map units, as well as plasmids pRB3464 and pRB3448 (with and without the HSV ICP4 gene, respectively), both encoding a G418 resistance gene under the control of the HSV thymidine kinase (TK) promoter, were obtained from Dr. Bernard Roizman (University of Chicago). The plasmid pD6P encoding an ICP6::*lac*Z fusion with intact ICP6 promoter region was provided by Dr. Sandra Weller (University of Connecticut Health Center; Goldstein and

Weller, 1988b). The plasmid pJF34, a vaccinia shuttle plasmid (pSC11) containing the UL37 gene under control of a vaccinia promoter, was constructed by Dr. Michael Pensiero (USUHS). The plasmid pJF14, containing *lac*Z within a micro-Mu cassette, was generated in the laboratory of Dr. Frank Jenkins and served as a source of the *lac*Z fragment for use in constructing *lac*Z fusions. All recombinant plasmid molecules generated for use in this study are described in Results, Section IV.

Bacterial transformation

Bacteria were made competent by the standard Hanahan method (1983). In this study, only *Escherichia coli* strains DH5α (BRL) and GM119 (dam/dcm mutant) were used. The competent cells were flash frozen in 0.5-1 ml aliquots via dry ice-ethanol bath and stored at -70°C. Each aliquot was thawed on ice prior to use. Competent cell preparations were tested by transformation with 1 μl of a test plasmid, closed circular DNA, usually pUC19, to determine competency efficiency.

Bacterial transformation was achieved by addition of either closed circular plasmid DNA or 1/3 of a diluted ligation mixture to 200 μl of competent cells and incubation on ice for 30 min. Typically, a 20 μl ligation mixture was diluted in transformation buffer (87.5 mM $CaCl_2$, 51 μM NaCl, 56.1 μM Na citrate, pH 7.0) to a volume of 300 μl just prior to bacterial transformation. The cells were then heat

shocked for 2 min at 42-45°C and placed again on ice for 2-5 min. Then, 800 μl of S.O.C. medium (LB with 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose), pre-warmed to 37°C, was added and the cells incubated at 37°C with shaking for 1.5 hr. Finally, 100 μl was spread on bacterial plates supplemented with the appropriate antibiotic (usually Ap plates). In some cases, plates contained 2% (v/v) X-gal and 25 mg/ml IPTG (Ap/Xgal/IPTG plates) for screening of β-galactosidase$^+$ (blue) versus β-galactosidase$^-$ (white) colonies. Plates were incubated overnight at 37°C in a non-CO_2 incubator.

Recombinant constructions

Restriction enzyme digestion of DNA

Plasmid DNA was diluted in buffers supplied by the manufacturer for digestion by restriction enzymes. Enzymes and buffers used in the various cloning procedures were purchased from either Boehringer Mannheim Biochemicals, Life Technologies, Inc. (BRL), Stratagene, New England Biolabs, or Promega. The amount of enzyme used, temperature, and time of digestion were as recommended by the manufacturer's protocol. Typically, 10-20 U/μg DNA were used in the smallest total volume possible, usually 10-20 μl. Double digests were performed simultaneously if the buffer for the restriction enzymes was the same. If the recommended reaction conditions differed only by salt concentration, then restriction enzyme digestions were performed sequentially, beginning with the enzyme and buffer with

lower salt, and then diluting out the initial buffer with H_2O and adding the second enzyme and buffer to complete the second enzymatic digest. Otherwise, restriction enzyme digestions were performed sequentially with phenol/chloroform extraction and ethanol precipitation before the second restriction enzyme digestion. Following restriction enzyme digestion, the reactions were either stopped by the addition of 10X loading buffer at a 1/10 dilution and individual fragments separated by agarose gel electrophoresis as described (see agarose gel electrophoresis) or, if the plasmid DNA was only linearized for further manipulation, the DNA was phenol/chloroform extracted followed by chloroform extraction and ethanol precipitation, resuspending in the appropriate buffer for the subsequent reaction (see Molecular cloning of DNA fragments, below).

Purification of DNA fragments

Plasmid DNA digested with restriction enzymes were separated by agarose gel electrophoresis. Once bands were visualized by ethidium bromide staining, the fragment of interest was cut out of the gel using a razor blade, cut into small pieces, and purified from the gel either by electroelution or using the Geneclean kit (Bio 101, Inc.).

Electroelution using an IBI electroelution apparatus (International Biotechnologies, Inc.) involved filling the rig with 500 ml 1X TBE and the V-shaped wells with 7.5M

ammonium acetate containing bromophenol blue, carefully removing all air bubbles with a pasteur pipette, and then placing the gel pieces in each separation well. The DNA was then electrophoresed into the ammonium acetate for 1 hr at 100 V. The ammonium acetate containing the DNA was removed by a syringe and precipitated by addition of 1 µl glycogen and an equal volume of isopropanol, placing at -80°C for 10-20 min, and DNA recovered by centrifugation in a microfuge for 5 min. The DNA pellet was resuspended in 200 µl TE and further purified by phenol/chloroform extraction followed by chloroform extraction and ethanol precipitation, resuspending the DNA pellet in 20 µl TE.

Purification of DNA fragments by Geneclean kit was performed as described by the manufacturer. This method involved dissolving the gel in 6M NaI and then binding the DNA to silica beads (glassmilk) at 4°C, washing several times, and eluting bound DNA at 45°C into TE. An aliquot of the total sample was electrophoresed on an agarose gel and ethidium bromide-stained to determine the percent recovery and purity.

Molecular cloning of DNA fragments

The strategy for generation of recombinant plasmids varied. In most cases, a DNA fragment was generated with restriction enzyme digestions that resulted in ends that were compatible with unique restriction enzyme sites within the vector. When necessary, DNA fragments that had

protruding 5' ends were changed to blunt-ended by a Klenow fill-in reaction for 30 min at room temperature which consisted of 4 μl 10X fill-in buffer (500 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 10 mM dithiothreitol [DTT], 500 μg/ml bovine serum albumin [BSA]), 2.5 μl each dNTP, 2 μl Klenow fragment, and 1 μg DNA diluted to 40 μl total volume by H_2O. The reaction was stopped by the addition of 2 μl 500 mM EDTA, deproteinized by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in 20 μl TE. Additionally, linkers could be ligated by adding a 20-fold molar excess of linkers over total 5' ends of target DNA and 1-2 μl T4 DNA ligase (3 U/μl, Promega) at 15°C overnight. To obtain a single linker insertion, the DNA was then digested with the appropriate restriction enzyme, and purified from the excess linkers by agarose gel electrophoresis. To prepare linkers for ligation, the 5' ends were phosphorylated by a kinase reaction consisting of 5 μg of the double-stranded oligonucleotide (1 μg/μl), 2 μl 10X kinase buffer (BRL), 3 μl T4 polynucleotide kinase (5 U/μl), 1 μl 10 mM dATP, and H_2O in a total volume of 20 μl, incubated at 37°C for 45 min, heat inactivated at 60°C for 10 min, and stored at -20°C. In this study, EcoRI linkers were used which were synthesized by M.F. Flora, USUHS.

Before ligation of a blunt-ended fragment with a blunt-ended vector, the ends of the vector were dephosphorylated

in order to prevent re-ligation of the vector upon itself. The dephosphorylation reaction consisted of 1-2 μg vector DNA, 5 μl 10X CIP buffer, 0.1 U calf intestinal phosphatase, and H_2O in a total volume of 50 μl, incubated at 56°C for 30 min. The reaction was stopped by adding 2 μl 0.5M EDTA. The DNA was deproteinized by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in 20 μl TE. After all the various manipulations to prepare fragment DNA and vector DNA, a small sample of each DNA was visualized by agarose gel electrophoresis to confirm the integrity of the DNA prior to ligation.

Typically, ligation reactions included 80-100 ng prepared vector and varying amounts of fragment, including a molar ratio of 1:2-5, 4 μl 5X ligase buffer (Promega), 1-2 μl ligase (3 U/μl, Promega), and H_2O in a total volume of 20 μl, incubated overnight at 15°C. Additionally, two negative control reactions were set up, one without ligase and without fragment to determine if the vector was completely digested and one with ligase and vector DNA only to determine the degree to which the vector was able to ligate upon itself. The ligation mixtures were diluted in transformation buffer and bacterial transformation was performed as described above.

Identification of recombinant plasmids

Bacterial colonies were screened by picking white

colonies (see Bacterial transformation) since the β-galactosidase gene is inactivated by the insertion of a DNA fragment in the multiple cloning site of most of the vectors used in this study. For vectors that did not contain this screening system, all colonies were picked. If the number of colonies was low, restriction enzyme digestions of small scale plasmid preparations was the method of choice to identify recombinant plasmids. Otherwise, 50-300 colonies were picked and patched on two bacterial plates supplemented with the appropriate antibiotic, one with a circle of Biodyne membrane placed directly on the agar surface for colony blots. In cases where most of the colonies were expected to contain recombinant plasmids (where the control plate containing the vector alone demonstrated no growth) or the colonies were few in number, the toothpick used to pick each colony was used to inoculate 3 ml LB for small scale plasmid preparations. Bacterial plates and liquid cultures were incubated overnight. The next day, the bacterial plates were stored at 4°C and no more than 60 plasmid DNAs were prepared according to the small scale plasmid preparation procedure described previously. 5 μl of each plasmid preparation was digested with the appropriate enzyme in a total volume of 10 μl to identify the recombinant plasmid and electrophoresed on a large agarose gel overnight. Additional restriction enzyme digestions were performed to confirm each positive clone.

To screen a large number of colonies grown on biodyne membranes, bacteria were lysed for DNA hybridization (colony blot) by passing each biodyne circle sequentially over a series of saturated 3M Whatman filters: (1) 0.5M NaOH, 0.5M NaCl for 10 minutes, (2) H_2O 1-2 min, (3) 1M Tris (pH 7.4), 1.5M NaCl for 5 min, (4) 1M Tris (pH 7.4), 1.5M NaCl for 5 min, (5) 2X SSC for 2 min, and (6) 2X SSC for 2 min. As a positive control, 1 μl of plasmid or fragment DNA to be used as the probe was spotted directly on the edge of each circle. Biodyne membranes were then baked for 2 hr at 80°C or UV crosslinked at 1200 microjoules using a Stratagene UV crosslinker followed by baking for 30 min at 80°C. Desired colonies were then detected by DNA hybridization (see Southern blots) with a radiolabelled DNA fragment probe. Bacterial colonies identified as positive by colony blot were then picked for inoculation of 3 ml overnight culture and small scale plasmid preparation and confirmed by restriction enzyme digestion of the plasmid DNA.

Southern blots

DNA fragments were transferred to either nitrocellulose or biodyne membranes for DNA hybridization with radiolabelled DNA fragment probes (Southern, 1975). Following agarose gel electrophoresis, ethidium bromide staining, and photography including a ruler to determine the fragments' distance of migration from the wells, gels were sequentially treated with three solutions prior to transfer.

First, acid depurination of the DNA was achieved by soaking the gel in 0.25M HCl for 15 min with gentle rocking and then the solution was replaced for a second treatment. The gel was then washed in deionized H_2O for 10 min with rocking two times. Second, alkaline denaturation was achieved by soaking the gel in 0.5M NaOH, 1M NaCl for 15 min with rocking two times. Third, the gel was neutralized by soaking in 0.5M Tris (pH 7.4), 3M NaCl for 0.5-1 hr with rocking two times. The gel was then placed on a sheet of 3M Whatman filter paper saturated with 5X SSC which served as a wick with its ends extending into a pan of 5X SSC. All air bubbles were removed. Nitrocellulose cut to the size of the gel was pre-soaked in H_2O and then carefully placed on top of the gel, again removing all air bubbles, followed by two pieces of 3M Whatman filter paper and a stack of paper towels. A weight was placed on top and the SSC was allowed to absorb up through the gel overnight into the paper towels about 1/2 of the way through the stack. The membrane was then baked at 80°C for 1.5-2 hr.

The blot was prehybridized overnight in a sealed plastic bag in 10 ml of 30% (v/v) formamide, 6X SSC, and 1X Denhardt's (1% [w/v] ficoll, 1% [w/v] polyvinylpyrrolidone, 1% [w/v] BSA) at 68°C. The blot was then hybridized in fresh hybridization solution with the addition of the radiolabelled DNA probe (5×10^5 to 1×10^6 cpm/ml hybridization buffer). The radiolabelled DNA probe was

prepared by random hexamer primer method (Feinberg and Vogelstein, 1983). The labelled DNA was separated from the free label by a G-50 spin column as described by Maniatis *et al* (1982). The probe was denatured by boiling for 2 min prior to addition to the hybridization buffer and mixed by vortexing. Prehybridization buffer was replaced with the hybridization buffer plus probe and the bag re-sealed. Hybridization was performed at 68°C overnight, and then the blot was removed and washed in 2X SSC, 0.1% (w/v) SDS for 15 min, four times, rocking at room temperature, and then two times, 0.5-1 hr, by 0.1X SSC, 0.1% (w/v) SDS in a sealed plastic bag at 68°C. The blot was then air dried on a paper towel, wrapped in cellophane, and subjected to autoradiography on Kodak XAR-5 film with an intensifying screen or put on a phosphorscreen. The size of each DNA fragment identified by Southern blot was determined by measuring the distance of migration from the well and comparison with the photograph of ethidium bromide-stained fragments. Dot blots for cellular DNA and colony blots for bacterial DNA were prehybridized, hybridized, and washed in a similar fashion.

<u>In vitro transcription</u>

The pHC37 plasmid DNA from large scale plasmid preparations served as the template DNA for *in vitro* transcription of the UL37 gene. The pHC37 plasmid DNA (10 μg) was linearized by digestion with EcoRI and purified

using Geneclean as previously described. Transcription of the linearized DNA from the T7 promoter was achieved using the Riboprobe system (Promega) according to the manufacturer's instructions. A small aliquot of the reaction mixture included radiolabelled UTP to determine the efficiency of the reaction. The reaction mixture included 1-2 μg linearized pHC37, 20 μl 5X transcription buffer, 10 μl 0.1M DTT, 2.5 μl RNasin, 5 μl each rATP, rCTP, rGTP, rUTP (rNTPs), 25 μl 7-methylguanosine triphosphate (to serve as a cap), 3 μl T7 polymerase, and diethylpyrocarbonate (DEPC)-treated H_2O in a total volume of 100 μl. An 8 μl aliquot was removed to a separate eppendorf tube and 2 μl ^{32}P-UTP (3,000 Ci/mmol) added prior to incubation. The unlabelled reaction mixture was incubated at 37°C for 90 minutes and the radiolabelled reaction for 0.5-1 hr. For each reaction mixture, the volume was completed to 100 μl with DEPC-treated H_2O and phenol extracted followed by chloroform extraction. The remaining organic phases were "back-extracted" with an equal volume of DEPC-treated H_2O. Final aqueous phases were combined and ethanol precipitated with 1/10 volume 2.5M ammonium acetate (pH 7.5) and 2 volumes ice-cold 100% ethanol, placed on ice for 15 min, and microfuged for 20 min. The radiolabelled RNA pellet was washed with 95% ethanol and resuspended in 20 μl DEPC-treated H_2O. The ethanol precipitation of the unlabelled RNA was interrupted prior to the centrifugation step and the

RNA stored at -20°C in ethanol. One-half of the radio-labelled RNA was electrophoresed on a formaldehyde/agarose gel as previously described to monitor the integrity and size of the synthesized RNA.

In vitro translation

RNA samples from *in vitro* transcription experiments were pelleted from ethanol and resuspended in 50 μl 10 mM Hepes (pH 7.4), and 5 μl was removed to measure the concentration by absorbance at 260 nm. The measurement of RNA concentration was repeated prior to each *in vitro* translation reaction to ensure accuracy. Then, 1 and 2 μg quantities of RNA were removed to eppendorf tubes for individual *in vitro* translation reactions and the remaining RNA ethanol precipitated as described above to store the RNA at -20°C. The RNA was then translated using a rabbit reticulocyte kit (NEN Research Products) according to the manufacturer's instructions. Control reactions including either no RNA or kit-supplied yeast RNA were used as negative and positive controls, respectively. Each reaction mixture included RNA, 10 μl rabbit reticulocyte lysate, 13 μl pre-mixed cocktail, and H_2O in a total volume of 25 μl. The pre-mixed cocktail for each reaction contained 5 μl L-^{35}S-methionine, and 5.5 μl cocktail, 2 μl 1M potassium acetate, and 0.5 μl 32.5 mμ magnesium acetate. A 1 μl aliquot of each reaction mixture was spotted on Whatman 540 filters prior to incubation for measurement of

trichloroacetic acid (TCA)-precipitable counts. The reaction mixtures were incubated at 37°C for 60 min. Following incubation, 1 μl of each reaction was spotted on filters. TCA precipitation of labelled proteins was achieved by saturating the filters with 3% (v/v) H_2O_2 and then boiling for 5 min in a beaker containing a large volume of 5% (v/v) TCA. The filters were then washed with ethanol followed by acetone and allowed to dry. Each filter was counted in 10 ml beta-fluor in a Beckman LS7500 scintillation counter. Efficiency of translation by ^{35}S-methionine incorporation was estimated by comparing the TCA-precipitable counts before and after each reaction. 10 μl of each translation reaction was electrophoresed on SDS-PAGE minigels as previously described. The gel was Coomassie stained and dried. ^{35}S-labeled proteins were detected by autoradiography on Kodak XAR-5 film.

Antibody production in rabbits

Generation of rabbit polyclonal antiserum against the UL37 protein was attempted by inoculation of three different antigens including UL37 *in vitro* translated proteins, a UL37 C-terminal peptide, and a purified UL37 vaccinia recombinant (V37). The UL37 *in vitro* translation reaction described above was upscaled 32-fold with replacement of labelled methionine with unlabeled L-methionine (Sigma) and served as the antigen for rabbit #487. Purified UL37 vaccinia recombinant (V37) was used to inoculate rabbit #651. The

UL37 C-terminal peptide (GHGPRQADREGA) prepared by Michael Flora (USUHS) was given to two rabbits (#S-1 & #634). The peptide was conjugated to keyhole limpet hemocyanin (KLH) by combining 10 mg peptide solubilized in 1 ml PBS and 10 mg KLH and 10 mg 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) in 9 ml PBS and mixing end-over-end overnight at 4°C. The conjugated peptide was then dialyzed against 2 L cold PBS two times for 2-3 hr each and stored at 4°C. Insoluble components were pelleted by centrifugation. Rabbit inoculations and serum collections were performed by Duncroft, Inc. (Lovettsville, Va). UL37 *in vitro* translated proteins (1 ml total volume) and C-terminal peptide were each emulsified with an equal volume of complete Freund adjuvant prior to inoculation.

Serum samples were screened by immunoblot of nitrocellulose strips containing electrophoresed infected cell proteins of HSV-1(F). An SDS-PAGE minigel using a preparative comb was run with a load of 0.5 ml of HSV-infected cell protein extract, 24 HPI, (see Preparation of infected cell protein extracts) in SDS-PAGE sample buffer. Following transfer to nitrocellulose, the miniblot was cut into strips using a razor blade. For initial screening of rabbit sera, each bleed was diluted 1/10 in 1% (w/v) nonfat milk in WB2 in a total volume of 750 μl for probing of each strip. Immunoblots were then performed as described previously using ^{125}I-labelled protein A for detection.

Other antisera

Mouse monoclonal antiserum directed against glycoprotein C (gC) and polyclonal antiserum directed against gD of HSV-1 were obtained from Dr. Barry Rouse (University of Tennessee, Knoxville) and Dr. Paul Kinchington (USUHS), respectively. Rabbit polyclonal antisera directed against the HSV-1 UL42 and ICP8 proteins were obtained from Dr. William Ruyechan (USUHS). Mouse monoclonal and rabbit polyclonal antisera, designated "α 1.1", directed against a UL37/maltose binding protein (MBP) fusion protein, containing the carboxy-terminal 1/3 of the UL37 protein, were obtained from Allen Albright (USUHS). Mouse monoclonal antiserum directed against actin was purchased from Chemicon International, Inc.

Preparation of infected cell protein extracts

For immunoblot analysis, CV-1 cells were infected with either HSV-1(F), HSV recombinant virus, or vaccinia recombinant virus at an MOI 10. At various times post infection, the cells were scraped, pelleted, washed 2-3 times with PBS, and solubilized in 1X sample buffer containing 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), 5% glycerol, and 0.15 mM bromophenol blue. Mock-infected CV-1 cells were used as a negative control. For each time point, 1.4 X 10^7 cells were infected, and extracts were prepared in a total volume of 2 ml, aliquoted, and stored at -20°C. Protein extracts were

sonicated and boiled for 2 min prior to electrophoresis. For studies involving the DNA synthesis inhibitor phosphonoacetic acid (PAA), cells were infected and maintained in the presence of PAA at 300 μg/ml.

For nuclear and cytoplasmic extracts, 1.4 X 10^7 CV-1 cells were infected with HSV-1(F), V37, or d21 at an MOI of 5 and harvested at 24 hours postinfection (HPI). In some experiments, the infected cell proteins were metabolically labelled with ^{35}S-methionine from 12 to 24 HPI by replacing the media with methionine-reduced media (9 parts methionine-free EMEM supplemented with glutamine plus 1 part EMEM-10%SP) with added ^{35}S-methionine at 50 μCi/ml. Cells were scraped, pelleted, washed in PBS, and the whole cell pellet quick frozen and stored at -70°C. To prepare nuclear and cytoplasmic extracts, each cell pellet was resuspended in 1 ml TE and placed on ice for 15 min, and then cells were lysed by 50 μl 10% (v/v) Nonidet P-40 (NP40) and then replaced on ice for 15 min. Nuclei were pelleted by centrifugation in a Beckman SS34 rotor at 5,000 rpm for 5 min at 4°C. The resulting supernatant served as the cytoplasmic infected cell protein extract. The nuclear pellet was resuspended in 1 ml TE, and sonicated for 30 s when necessary, resulting in a homogeneous nuclear infected cell protein extract. Protease inhibitors were added to each nuclear and cytoplasmic infected cell protein extract at a final concentration of 10^{-5}M TPCK, 10^{-5}M TLCK, and

aprotinin diluted 1:100. Then, 75 μl of each sample was removed and combined with 25 μl 4X SDS-PAGE sample buffer for immunoblot analysis. The remainder of each sample was stored at 4°C for use in immunoprecipitation studies. In one experiment, the whole cell pellet and nuclei prepared from HSV-1(F), V37, and d21 infected cells were treated with a high salt buffer (1.7M NaCl in TE plus protease inhibitors) for 40-60 min on ice to lyse the nuclei and inhibit DNA-binding by infected cell proteins. The insoluble protein and nucleic acid was then pelleted by centrifugation in a Beckman SS34 rotor at 19,000 rpm for 30 min at 4°C. The samples were then dialyzed twice overnight against 1 L TE supplemented with 12 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor. The final volumes of both cytoplasmic and salt-treated nuclear extracts were completed to equivalent volumes (2 ml) with TE supplemented with the group of protease inhibitors described above and samples were used for immunoprecipitation studies.

For single-stranded (SS) and double-stranded (DS) DNA-binding studies, protein extracts were prepared essentially as previously described (Powell & Purifoy, 1976). For each preparation, 10-20 150 cm^2 flasks of confluent CV-1 cells were infected at a multiplicity of 5-10 pfu/cell. At 24 HPI, the cells were scraped, pelleted, and quick frozen at -70°C. Prior to use, the cell pellet was lysed gently on ice in 10 ml of high salt buffer (HSB) containing 50 mM

Tris-HCl (pH 7.6), 5 mM EDTA, 0.5 mM DTT, 0.12 mM PMSF, and 1.7M KCl. The nucleic acid was removed by centrifugation at 35,000 rpm in a Beckman Ti50 rotor for 2 hr at 4°C. The remaining supernatant was dialysed overnight two times against 2 L of TEDGP buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM EDTA, 0.5 mM DTT, 20% (v/v) glycerol, 0.12 mM PMSF, and 100-150 mM KCl (equal to the the salt concentration used for equilibrating the column prior to column chromatography). The insoluble material was pelleted by centrifugation for 30 min in a Beckman SS34 rotor at 19,000 rpm prior to loading. In one experiment, the insoluble fractions were combined and solubilized by sonication and boiling in 1X sample buffer for SDS-PAGE and immunoblot.

The infected cell protein preparation for isoelectric focusing was prepared in the same manner as for DNA-binding studies except proteins were dialyzed against EDGP (the same solution as TEDGP without buffer or salt).

Virion purification

Extracellular virions were isolated essentially as described by Powell and Watson (1975). Vero or CV-1 cells were infected with HSV-1(F) at an MOI of 10. At 24 HPI, infected cells were scraped into the medium and pelleted by low-speed centrifugation. The resulting supernatant was clarified by a second centrifugation at 3,000 X g for 10 min. The supernatant containing released virus was then

centrifuged at 12,000 X g for 3 hr at 4°C. The pellet was resuspended overnight in TE. After brief sonication and centrifugation at 2,000 rpm for 5 min, the supernatant was layered onto a 36 ml 5-40% (v/v) sucrose gradient and centrifuged in a Beckman SW28 rotor at 13,000 rpm for 45 min at 4°C. The virus band was collected and the sucrose diluted out 1:5 with TE. Virions were pelleted by centrifugation in a SW28 rotor at 25,000 rpm for 1 hr at 4°C. The virus pellet was resuspended in a small volume of TE.

SS and DS DNA column chromatography

Single-stranded (SS) and double-stranded (DS) DNA column chromatography were performed as described by Purifoy and Powell (1976). SS DNA agarose (BRL) and DS DNA cellulose (Pharmacia) were equilibrated and packed as 5-10 ml columns using column buffer, either 100 or 150 mM KCl in TEDGP. Elimination of nonspecific binding was achieved by blocking with 10 column volumes of BSA 500 μg/ml in column buffer. Columns were re-equilibrated with 10 column volumes of column buffer prior to each column run. For column chromatography, each column was loaded with the infected cell protein preparation, washed with 10 column volumes of column buffer, and then bound proteins were eluted using a 50 ml linear KCl gradient, 100 or 150 mM to 1M KCl in TEDGP. Columns were then stripped using a 2M KCl-TEDGP step. Fractions, 2 ml each, were collected continuously from the

time infected cell proteins were loaded through the 2M step. A small aliquot of each fraction was combined with SDS-PAGE sample buffer for immunoblot analysis. In one experiment, individual fractions were assayed by Dr. William Ruyechan's laboratory for HSV DNA polymerase activity under high-salt (100 mM KCl) conditions specific for the viral enzyme as previously described (Ruyechan and Weir, 1984).

Isoelectric focusing

Preparative isoelectric focusing of HSV-1(F) and V37 infected cell proteins was achieved using a Bio-Rad Rotofor cell according to the manufacturer's instructions. Prior to each run, the anion and cation exchange membranes were equilibrated overnight in 0.1M NaOH and 0.1M H_3PO_4, respectively. Once the Rotofor cell was assembled and connected to a water-cooling apparatus, the electrode chambers were filled with 25 ml of fresh electrolyte and the focusing chamber filled with distilled deionized H_2O for a pre-run at 12W constant power to eliminate electrolyte leakage. The pre-run was repeated until the current was reduced to less than 6 mA. The focusing chamber was then emptied, re-sealed, and refilled with the diluted load (55-60 ml) consisting of prepared infected cell proteins and 2% (v/v) Bio-lyte (Bio-Rad) in fresh EDGP (see Preparation of infected cell protein extracts). The Rotofor was run at 12W constant power for 6 hr. Generation of a completed pH gradient was observed by an increase in voltage to 800-1000V

and a decrease in current. Fractions were then harvested and stored at 4°C. A small aliquot of each fraction was combined with SDS-PAGE sample buffer for immunoblot analysis.

Immunoprecipitation

Immunoprecipitations were performed essentially as described by Harlow and Lane (1988). Unlabelled or radiolabelled lysates from HSV-1(F), V37, and d21-infected cells (see Preparation of infected cell protein extracts) and specific antiserum were combined and mixed end-over-end at 4°C overnight. The antiserum required to immunoprecipitate the protein(s) of interest was titrated with each lysate preparation and, typically, 200 μl of lysate and 10 μl of antiserum were optimal. A reaction using normal rabbit serum was used as a negative control. The immune complexes were next incubated end-over-end for 2-3 hr at 4°C with 100 μl of 10% (v/v) protein A-sepharose CL-4B (Sigma) in RIPA buffer (150 mM NaCl, 1% [v/v] NP40, 0.5% [w/v] deoxycholate, 0.1% [w/v] SDS, 50 mM Tris-HCl, pH 7.5) supplemented with a group of protease inhibitors including 10^{-5}M TPCK, 10^{-5}M TLCK, and aprotinin diluted 1:100. The immunoprecipitates were then washed 4 times in ice-cold supplemented RIPA buffer, using a 15 s microfuge spin for each wash, and the final pellet resuspended in 100 μl SDS-PAGE sample buffer. After boiling for 2 min and a 15 s microfuge spin, 20 μl of each supernatant was run on SDS-

PAGE. Gels containing radiolabelled proteins were Coomassie stained and dried. Gels containing unlabelled proteins were transferred to nitrocellulose for immunoblot analysis.

RESULTS

The aim of this dissertation was to identify and characterize the gene product encoded by the UL37 open reading frame (ORF) of HSV-1. The results of this study can be divided into three phases: (1) production of UL37-specific reagents, (2) identification and characterization of the UL37 protein in HSV-infected cells and virions, and (3) biochemical studies of the UL37 protein.

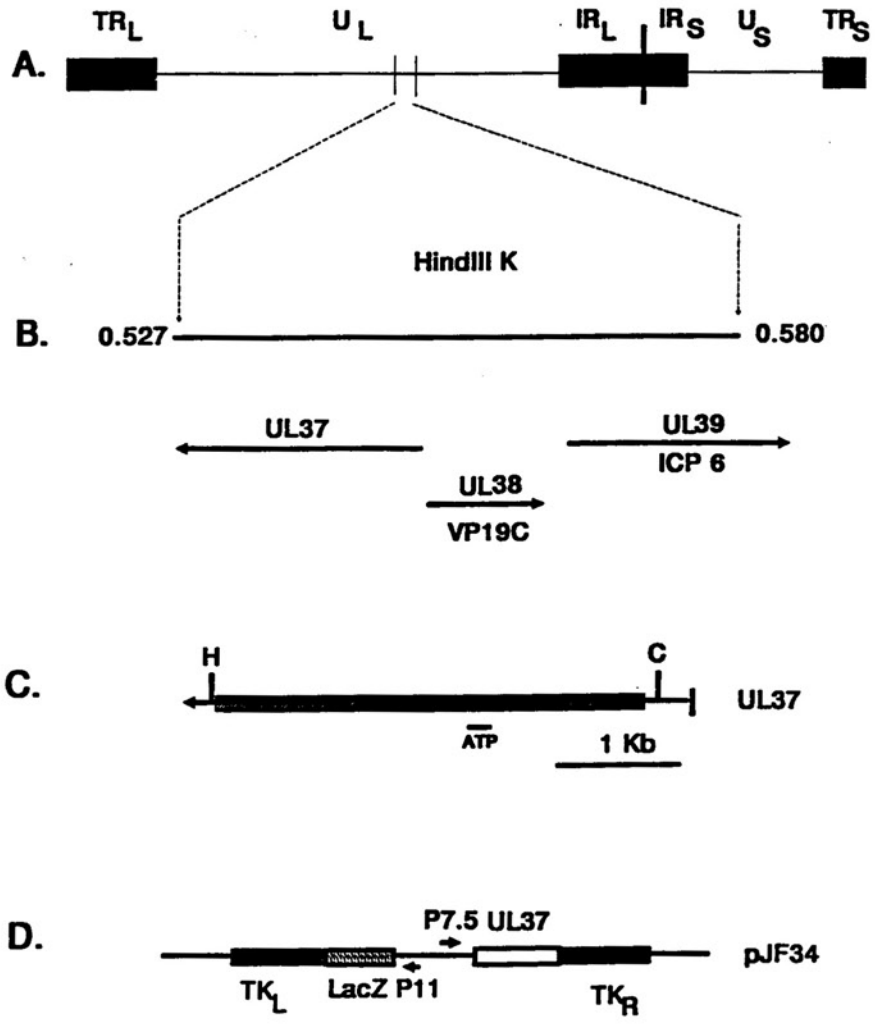
I. Production of UL37 specific reagents

Cloning and expression of the UL37 ORF

In order to express the UL37 protein in an *in vitro* system, the UL37 gene was cloned into a multipurpose plasmid vector, pBluescript SK+, which allows for *in vitro* transcription of the inserted gene by placing the gene under the control of either a T3 or T7 polymerase promoter depending on the orientation of the inserted gene. The plasmid pHC37 was constructed by cloning a 3.47 kb *Hin*dIII-*Cla*I DNA fragment from pRB210, containing the *Hin*dIII K fragment of HSV-1(F), into the *Hin*dIII-*Cla*I sites of pBluescript SK+ (Fig. 9, lines B & C). The *Cla*I site is located 92 bp upstream of the UL37 start codon. No ATGs are located between the *Cla*I site and the UL37 ATG. The *Hin*dIII site is located 3 bp downstream of the stop codon. This construct served as a template for *in vitro* transcription of UL37 utilizing the T7 promoter within the plasmid vector. Prior to the transcription reaction, pHC37 was linearized

FIGURE 9

Genomic location of the UL37 ORF and subclones used for UL37 expression. (A) Sequence arrangement of the HSV-1 DNA genome showing the location of the unique sequences of the L and S components (U_L and U_S) and the terminal (TR_L and TR_S) and inverted (IR_L and IR_S) repeats. (B) Schematic of the *Hin*dIII K fragment showing the locations and directions of transcription of the UL37, UL38, and UL39 genes. (C) Schematic representing the UL37 gene and *Hin*dIII-*Cla*I fragment which was subcloned for *in vitro* transcription. The hatched box indicates the UL37-coding region. H, *Hin*dIII; C, *Cla*I. The location of the potential ATP-binding domain is indicated by a solid bar. (D) Sequence arrangement of the vaccinia shuttle plasmid pJF34. Solid boxes indicate flanking vaccinia virus TK sequences. The hatched box represents the location of the β-galactosidase gene. The open box represents the location of the UL37 gene.


A.
TRL
UL
IRL
IRS
US
TRS
HindIII K
B.
0.527
0.580
UL37
UL39
ICP 6
UL38
VP19C
C.
H
C
UL37
ATP
1 Kb
D.
P7.5 UL37
pJF34
TKL
LacZ P11
TKR

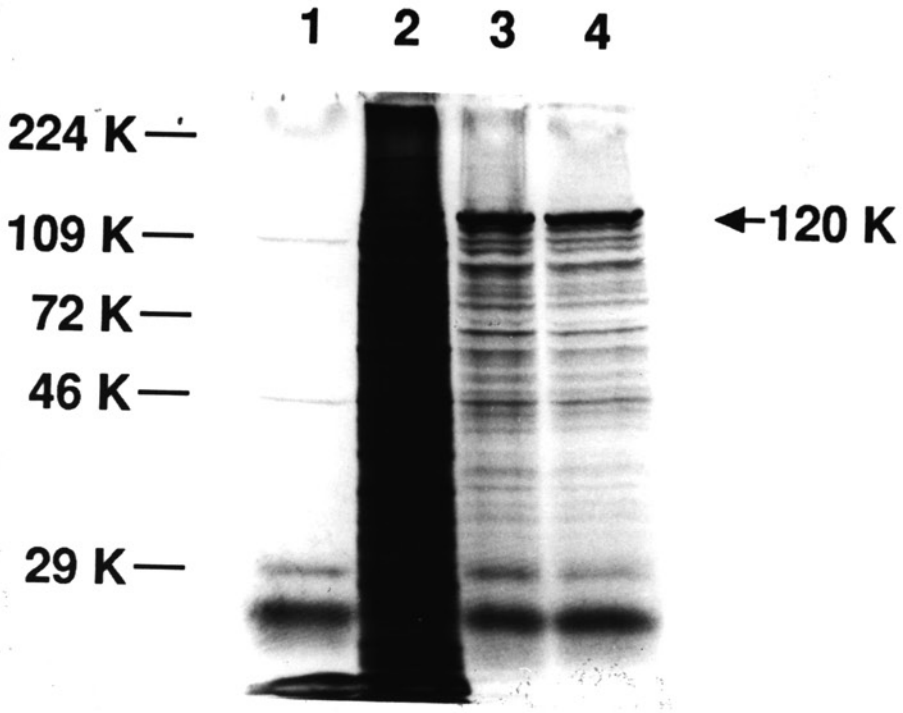
with EcoRI to terminate transcripts 17 bp downstream of the UL37 stop codon. *In vitro* transcription of pHC37 produced a single transcript of 3.5 kb (data not shown). This synthesized RNA served as a template for *in vitro* translation, producing a prominent 120 kD protein band on SDS-PAGE (Fig. 10, lanes 3 and 4) and a series of smaller bands most likely representing premature termination products. These results were in partial agreement with the findings of Anderson *et al* (1981) where *in vitro* translation of hybrid-selected mRNA from HSV-1-infected cells, mapping to the region of the UL37 ORF, produced a major 120 kD band and a minor 85 kD band. The observed size of the the *in vitro* translated UL37 protein, 120 kD, corresponds well with the size of the protein based on the predicted amino acid sequence, 120.5 kD.

UL37 expression in vaccinia virus

A recombinant vaccinia virus expressing UL37 was constructed to serve as a source of UL37 protein independent from other HSV proteins and to confirm the specificity of the UL37 antisera. The plasmid pJF34 was constructed by Dr. Michael Pensiero and was used to produce the UL37-expressing recombinant virus, V37. The plasmid pJF34 is a vaccinia shuttle plasmid, containing the UL37 ORF under the control of the vaccinia virus promoter, p7.5, and a β-galactosidase gene under the control of the p11 promoter. The β-galactosidase and UL37 genes are flanked by the

FIGURE 10

Autoradiogram of ^{35}S-methionine-labelled proteins generated by in vitro translation reactions. Input RNA was as follows. Lane 1, none; lane 2, control yeast RNA; lanes 3 and 4, *in vitro* translation products of pHC37-derived RNA. Proteins were separated on an SDS-10% polyacrylamide gel, fixed, and dried as described in Materials and Methods. The molecular sizes of protein standards are indicated on the left.


1
2
3
4
224 K
109 K
72 K
46 K
29 K
120 K

vaccinia virus TK gene (Fig. 9, line D). UL37 expression by V37 was confirmed by immunoblot analysis of V37-infected cell proteins using the UL37-specific antiserum, 487. As is discussed in the next section, V37 was found to produce a UL37 protein indistinguishable from the UL37 protein of HSV-infected cells by immunoblot analysis.

Production of UL37-specific antiserum

The *in vitro* translation reactions utilizing pHC37-derived transcripts as a substrate were scaled up 32-fold and used to produce the rabbit polyclonal anti-UL37 antiserum, 487. Immunoblot analysis of HSV-1 infected cell proteins using the 487 antiserum identified a single band with a molecular mass of 120 kD among HSV-infected cell proteins that was not present in mock-infected cells (Fig.11, left panel). The antibody specificity was confirmed by immunoblot analysis of V37, VSC11, and mock-infected cell proteins as seen in Fig. 12. The 487 antiserum reacted with a 120 kD vaccinia-expressed UL37 protein from two V37 isolates (V37-1 and V37-2), but not mock and VSC11-infected cells. VSC11 is a recombinant vaccinia virus that was constructed in the same manner as the V37 virus except that the vaccinia shuttle plasmid, containing a β-galactosidase gene, did not include the UL37 gene.

Other rabbit polyclonal antisera reactive with the UL37 protein in HSV-1 infected cells were generated by the

FIGURE 11

Immunoblot analysis of infected cell proteins probed with UL37-specific antisera. Proteins harvested at 24 HPI were separated by SDS-9% polyacrylamide gels, transferred to nitrocellulose, and probed with 487 antiserum (left) and 1.1 antiserum (right). Antibody binding was detected with ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. Apparent molecular size based on relative migration to high molecular weight protein standards (not shown) are indicated.

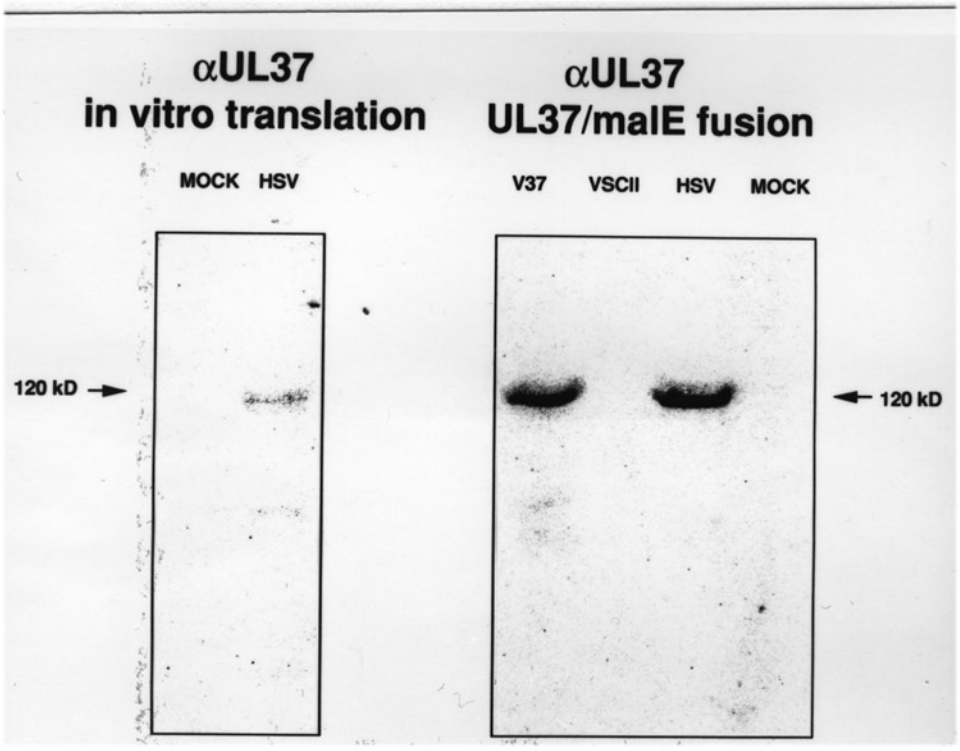
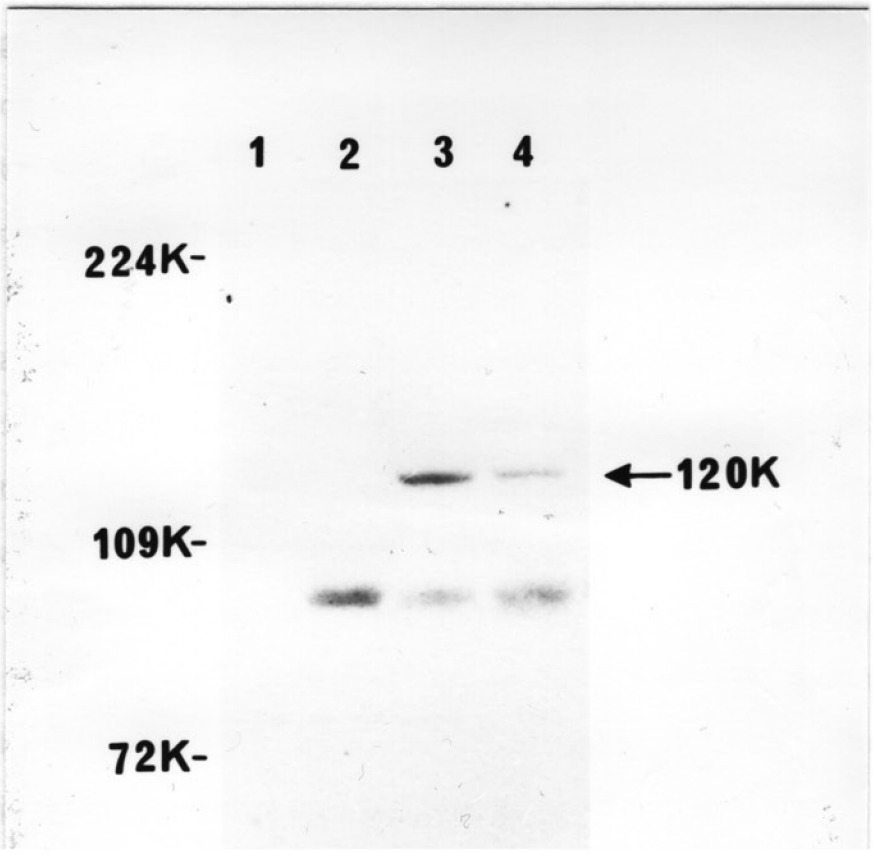

αUL37
in vitro translation
MOCK HSV
120 kD
αUL37
UL37/malE fusion
V37 VSCII HSV MOCK
120 kD

FIGURE 12

Autoradiogram of an immunoblot of vaccinia virus-infected cell proteins. Infected cell proteins harvested at 24 HPI were separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with 487 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. Lane 1, mock-infected cells; lane 2, VSC11-infected cells; lane 3, V37-1-infected cells; lane 4, V37-2-infected cells. The molecular sizes of protein standards are indicated on the left.

The V37 isolates were generated using the plasmid pJF34 (Fig. 9, line D), containing the UL37 gene under the control of the vaccinia p7.5 promotor. VSC11 was generated using the plasmid pSC11 which is identical to pJF34 except it does not contain the UL37 gene.


1
2
3
4
224K-
109K-
72K-
←120K

inoculation of the V37-1 recombinant (#651) and a UL37 C-terminal peptide (#S-1); however, the use of these antisera in the experiments reported in this study was limited since they demonstrated either high background or poor reactivity compared to the 487 antiserum.

Approximately two-thirds of the way through this study, mouse and rabbit polyclonal antisera (designated anti-1.1) directed against a fusion protein consisting of the *E.coli* maltose binding protein fused to the C-terminal 366 amino acids of the UL37 protein (approximately 1/3 of the UL37 protein) were generated within this laboratory. This 1.1 antiserum reacted more strongly against the UL37 protein from both HSV and V37-infected cells than the 487 serum in immunoblot analysis (Fig. 11, right panel) and was capable of immunoprecipitating the UL37 protein from HSV and V37-infected cells (Allen Albright, personal communication).

II. Analysis of UL37 in HSV-1 infected cells and virions

Expression of UL37 in HSV-1 infected cells

Since the major events occurring during HSV replication in cell culture have been defined, determining the relative amount of a protein present at different times postinfection can be a useful indicator of its potential role in viral replication. For example, α proteins which are expressed within 2-4 hours post infection (HPI) have been generally identified as regulatory. β proteins which are maximally expressed from 4-7 HPI are involved in DNA

replication, and γ proteins, expressed once DNA replication has been initiated, tend to be structural proteins. Kinetic studies were performed to determine at what stage of the HSV replication cycle the UL37 protein is present.

The expression of UL37 during a lytic HSV-1 replication cycle in infected cells was analyzed by determining the kinetics of appearance of the UL37 protein. The temporal class of HSV genes to which UL37 belongs was specified with the following kinetic data and experiments with viral DNA synthesis inhibitors. For kinetics of appearance, CV-1 cells were infected with HSV-1(F) at an MOI of 10, and infected cell protein extracts were prepared from individual flasks harvested at 3 hr intervals up to 24 HPI. The infected cell proteins were analyzed by immunoblotting with antisera directed against UL37 and two additional HSV-1 proteins. As internal controls for the individual time points, the protein extracts were probed with antisera directed against UL42, a 65 kD DNA binding protein (Gallo *et al*, 1988; Marsden *et al*, 1987), and ICP8, the major HSV-1 DNA binding protein (Bayliss *et al*, 1975; Powell and Purifoy, 1976; Purifoy and Powell, 1976). The kinetics of appearance in time course experiments for both of these proteins have been reported previously (Goodrich *et al*, 1989; Olivo and Challberg, 1988). Both the UL42 and ICP8 proteins appeared early, being detected by 6 HPI (Figs. 13 & 14). The ICP8 protein was detected as early as 3 HPI by

FIGURE 13

Autoradiogram of an immunoblot of HSV-1-infected cell proteins harvested at various times postinfection probed with anti-UL42 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at the times indicated and separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with a rabbit polyclonal anti-UL42 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the right.

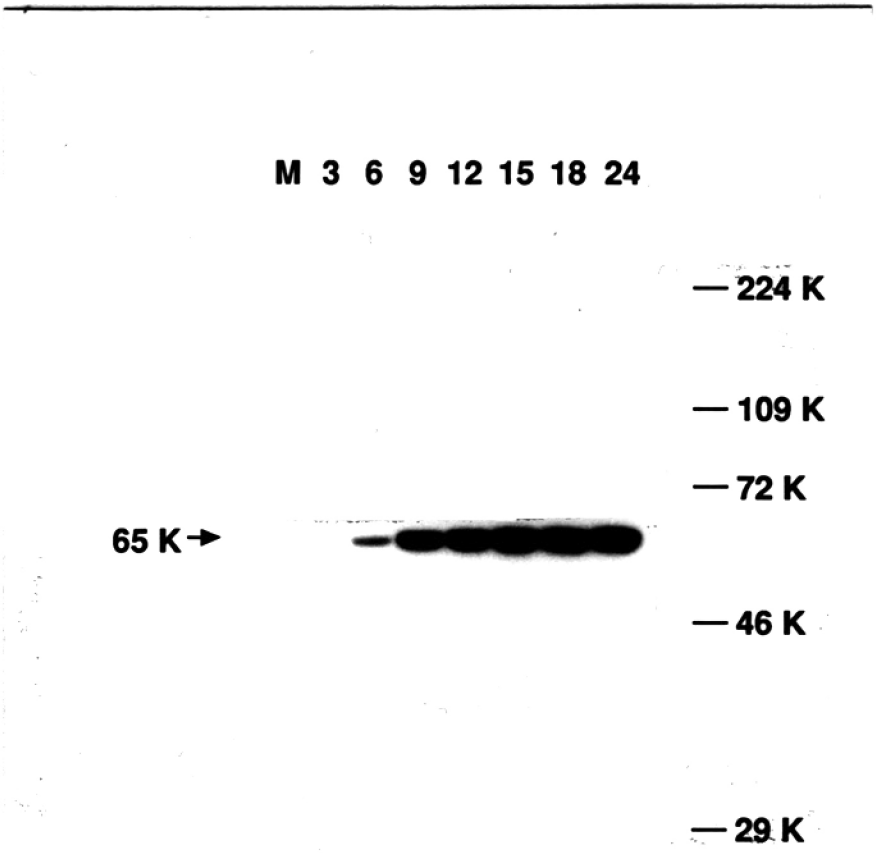
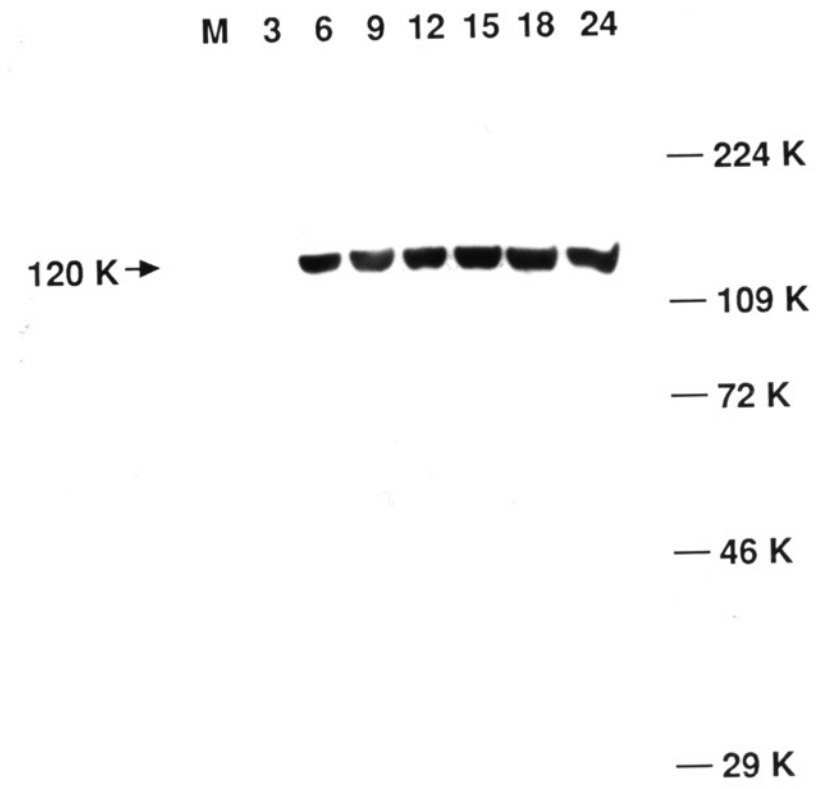

M 3 6 9 12 15 18 24
224 K
109 K
72 K
65 K
46 K
29 K

FIGURE 14

Autoradiogram of an immunoblot of HSV-1-infected cell proteins harvested at various times postinfection probed with anti-ICP8 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at the times indicated and separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with a rabbit polyclonal anti-ICP8 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the right.


M 3 6 9 12 15 18 24
— 224 K
120 K →
— 109 K
— 72 K
— 46 K
— 29 K

using a longer exposure of the immunoblot (data not shown). Both the UL42 and ICP8 proteins reached maximal levels by 9 to 12 HPI and maintained these levels throughout the entire replication cycle. These results are in complete agreement with the previous reports. In contrast, probing with the 487 antiserum, the UL37 protein appeared later in infection, being detected at 9 HPI, and gradually increased in amount, reaching its highest levels very late in infection, 24 HPI (Fig. 15). Similar analysis using the 1.1 antiserum also demonstrated peak UL37 accuumulation late in infection and, in addition, UL37 was detected as early as 6 HPI (Fig. 16). The results of this experiment clearly demonstrate that the kinetics of appearance of the UL37 protein during HSV-1 replication is distinct from the appearance of the UL42 and ICP8 proteins (Fig. 17).

Since the UL37 protein appears late in infection, it was reasoned that the gene may belong to either the $\gamma 1$ or $\gamma 2$ class of HSV genes, using the nomenclature of Roizman (Honess and Roizman, 1974; Roizman and Sears, 1990). The $\gamma 1$ class of HSV genes is defined as those genes whose transcripts are produced in small amounts in the absence of viral DNA synthesis, yet reach their maximum levels of synthesis after the onset of DNA replication, while the $\gamma 2$ class of HSV genes absolutely requires DNA synthesis for expression. To define precisely the temporal class to which UL37 belongs, infected cell proteins were harvested from

FIGURE 15

Autoradiogram of an immunoblot of HSV-1-infected cell proteins harvested at various times postinfection probed with 487 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at the times indicated and separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with 487 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the right.

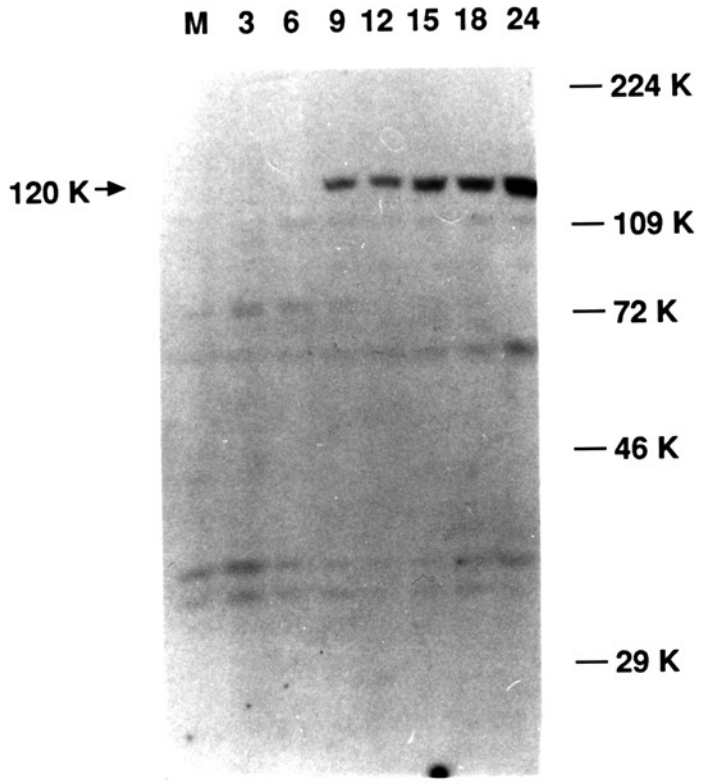

M 3 6 9 12 15 18 24
120 K
224 K
109 K
72 K
46 K
29 K

FIGURE 16

Immunoblot analysis of HSV-1-infected cell proteins harvested at various times postinfection probed with 1.1 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at the times indicated and separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with 487 antiserum. Antibody binding was detected with ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. The molecular sizes of protein standards are indicated on the right.

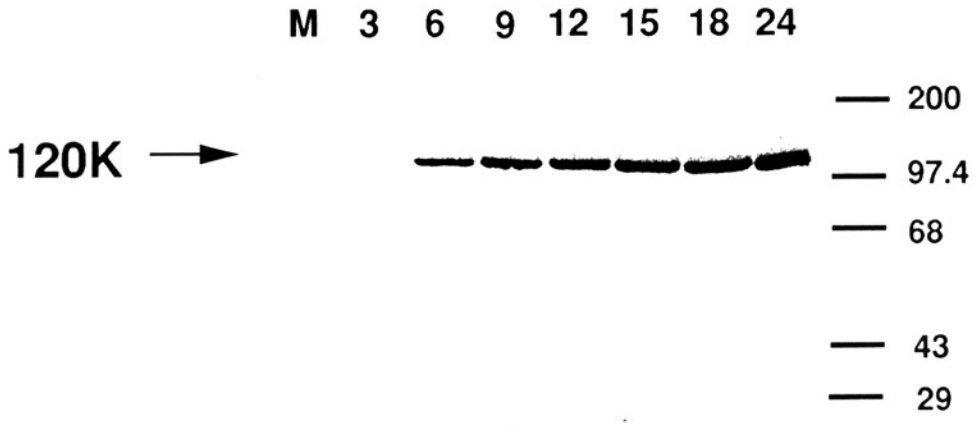
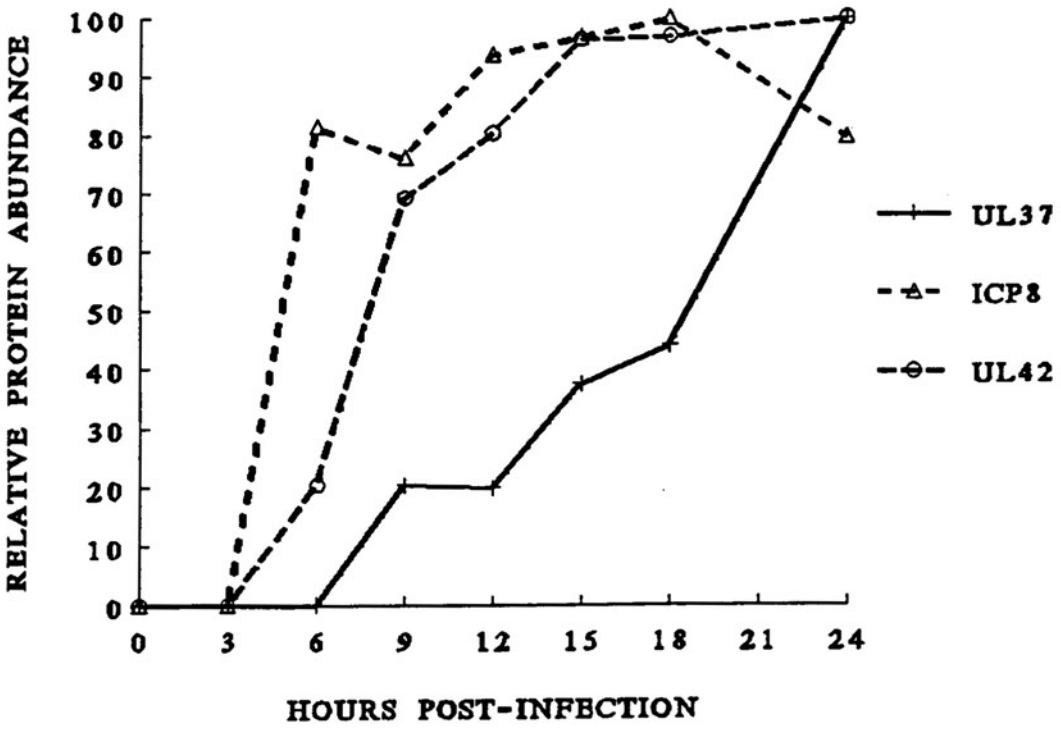

M 3 6 9 12 15 18 24
120K
200
97.4
68
43
29

FIGURE 17

Graph showing the kinetics of appearance of the UL37, ICP8, and UL42 proteins in HSV-1-infected cells. Bands from autoradiograms shown in Figs. 13, 14, and 15 were scanned densitometrically and data summarized in this graph. The values shown represent the relative abundance of the detected protein from each blot.


RELATIVE PROTEIN ABUNDANCE
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
18
21
24
HOURS POST-INFECTION
UL37
ICP8
UL42

CV-1 cells that had been infected and maintained in the presence of the DNA synthesis inhibitor, phosphonoacetic acid (PAA). The protein extracts were analyzed by immunoblotting using either 487 antiserum, antiserum directed against ICP8, or antiserum directed against gC of HSV-1. ICP8 has been previously shown to belong to the β1 class of HSV genes (Honess and Roizman, 1974), while gC has been shown to belong to the γ2 class (Frink *et al*, 1983). As a β gene, ICP8 was easily detected in both the presence and absence of PAA, with higher levels seen in the presence of PAA, whereas gC, a γ2 gene, was detected only in the absence of PAA (Figs. 18 & 19). In contrast, the UL37 protein was detected in both the presence and absence of PAA, with higher levels detected in the absence of PAA (Fig. 20). Two additional bands of 42 and 45 kD were detected when the PAA lanes were probed with the UL37 antiserum. While the identity of these bands is not known, their presence in both HSV and mock-infected lanes indicates that they represent cellular proteins to which the rabbit polyclonal antiserum cross-reacts. Their presence only in the PAA-treated cells suggests that they may represent cellular stress proteins induced by PAA. On the basis of the higher levels of the UL37 protein in the absence of PAA and the increased production of the protein late in infection, we have assigned UL37 to the γ1 class of HSV genes.

FIGURE 18

Autoradiogram of an immunoblot of infected cell proteins from cells grown in either the presence (+) or absence (-) of PAA probed with anti-ICP8 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at early (6HPI) and late (15 HPI) time points and separated on a SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-ICP8 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the right.

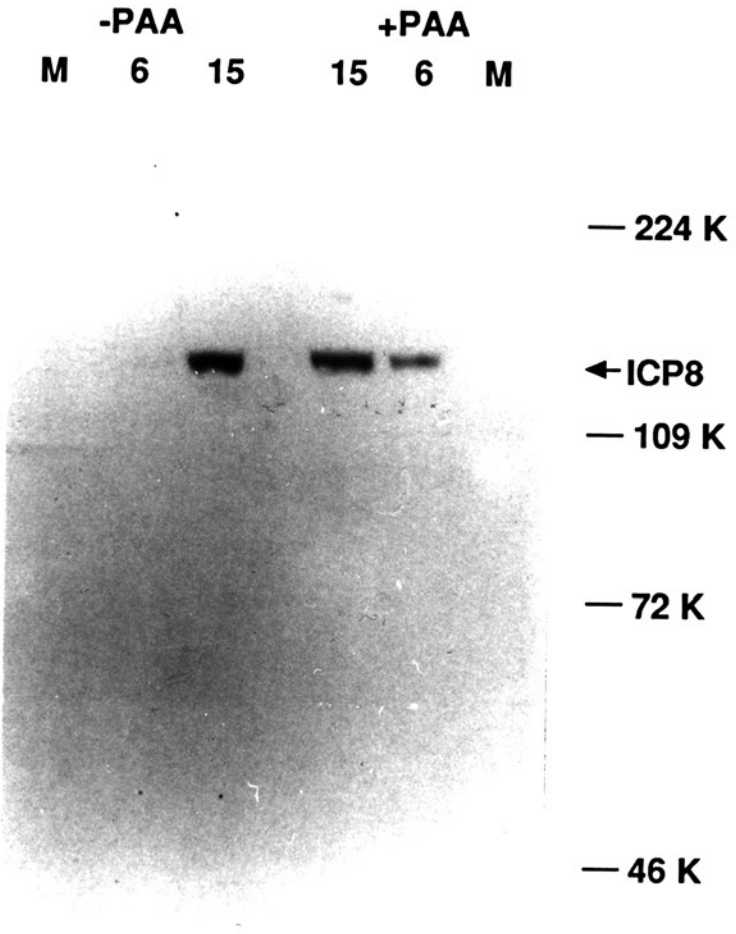

-PAA
+PAA
M
6
15
15
6
M
— 224 K
←ICP8
— 109 K
— 72 K
— 46 K

FIGURE 19

An immunoblot of infected cell proteins from cells grown in either the presence (+) or absence (-) of PAA probed with anti-gC antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at early (6HPI) and late (15 HPI) time points and separated on an SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with monoclonal anti-gC antiserum. Antibody binding was detected with biotinylated anti-mouse antiserum and Vectastain peroxidase system as described in Material and Methods. The molecular sizes of protein standards are indicated on the left. gC, glycoprotein C; pgC, precursor to mature gC.

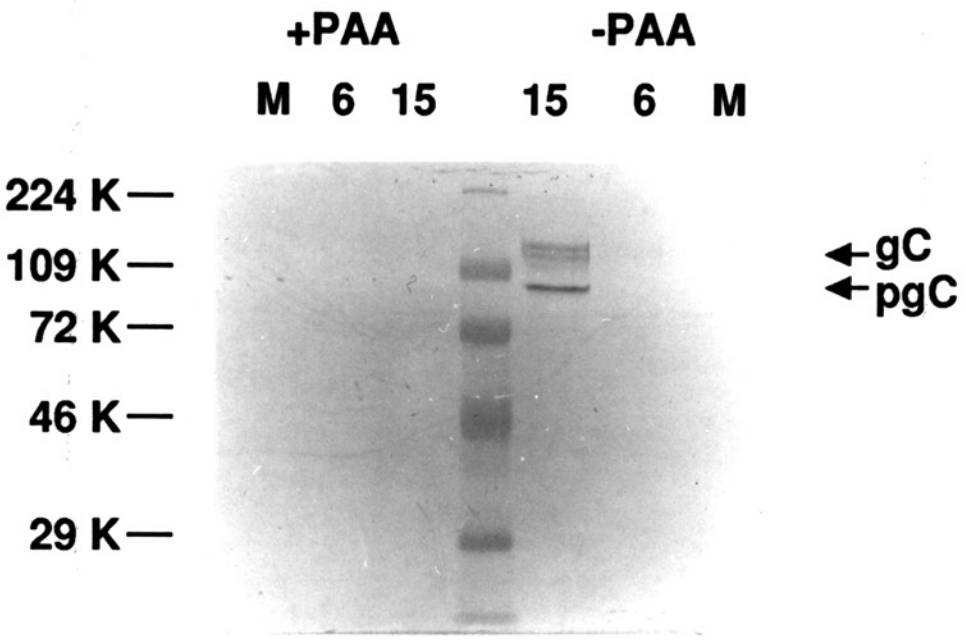
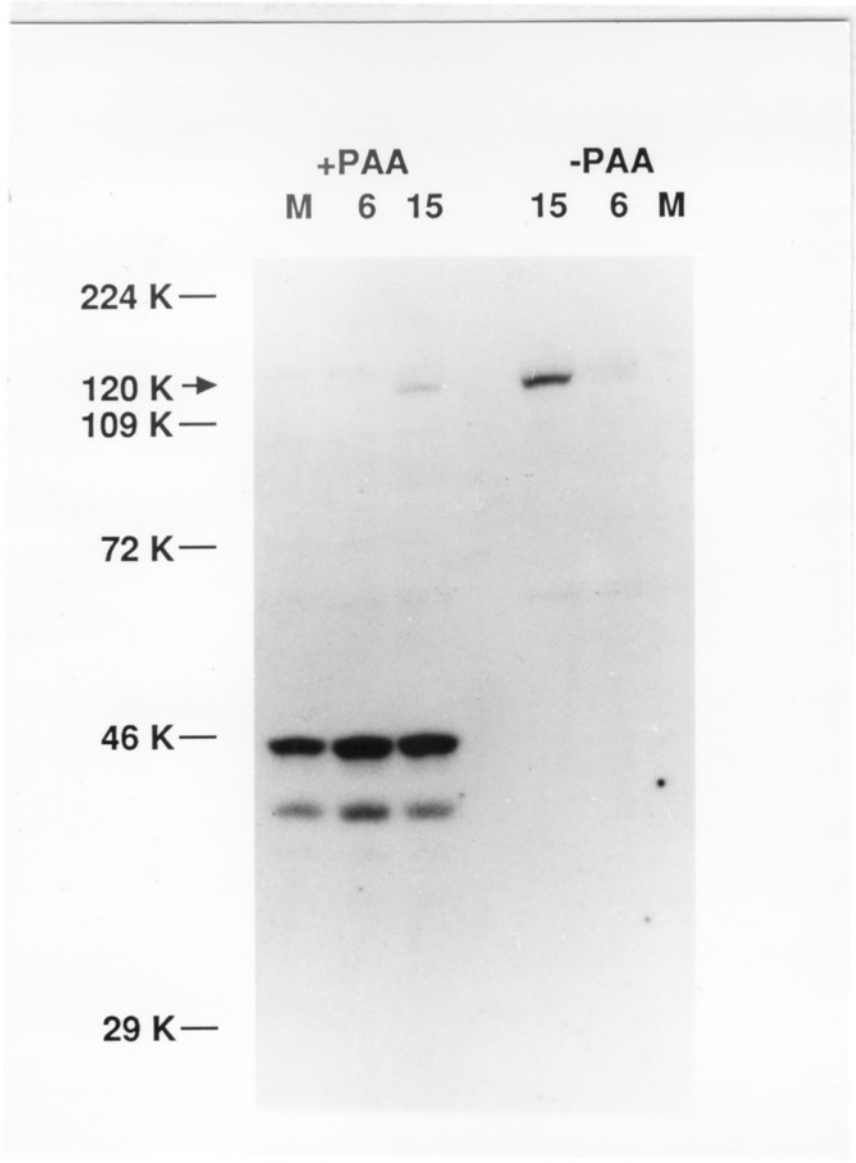

+PAA
-PAA
M 6 15 15 6 M
224 K—
109 K—
72 K—
46 K—
29 K—
gC
pgC

FIGURE 20

Autoradiogram of an immunoblot of infected cell proteins from cells grown in either the presence (+) or absence (-) of PAA probed with 487 antiserum. Proteins were harvested from mock-infected cells (M) and HSV-1-infected cells at early (6HPI) and late (15 HPI) time points and separated on an SDS-9% polyacrylamide gel, transferred to nitrocellulose, and probed with 487 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the left.


+PAA
-PAA
M 6 15
15 6 M
224 K
120 K
109 K
72 K
46 K
29 K

Analysis of HSV-1 virions

The assignment of UL37 to the γ1 class raised the possibility that this protein is a component of the HSV virion since the majority of the γ gene products represent structural proteins. To test this hypothesis, intact enveloped HSV-1 virions were isolated, and protein extracts from these virions were probed by immunoblotting with antisera directed against the UL37, ICP8, and gD proteins of HSV-1. The gD and ICP8 antisera served as controls for structural and nonstructural HSV proteins, respectively. A Coomassie stain of an SDS-polyacrylamide gel containing the separated virion proteins demonstrated a typical protein profile for purified HSV virions, as described previously by Spear and Roizman (1972), with no contamination with cellular proteins by visual examination (Fig. 21). Antiserum directed against ICP8, a known nonstructural HSV protein (Honess and Roizman, 1973), failed to detect ICP8 in the virion preparation, while easily detecting the presence of ICP8 in the infected cell protein extracts harvested at 25 HPI (Fig. 22). Antiserum directed against gD, a component of intact virions (Spear and Roizman, 1972; Heine *et al*, 1972), easily detected the mature form of the protein in the virion preparation and detected both the mature and precursor forms in the infected cell protein extracts (Fig. 23). Analysis probing with antiserum directed against UL37 failed to detect the presence of UL37 protein in HSV-1

FIGURE 21

Coomassie blue stain of proteins from purified HSV-1 virions. Virions were prepared as described in Materials and Methods. Virion proteins were separated on an SDS-9% polyacrylamide gel and stained with Coomassie blue. VP, virion preparation; MK, protein standards. The molecular sizes of protein standards are indicated on the right.

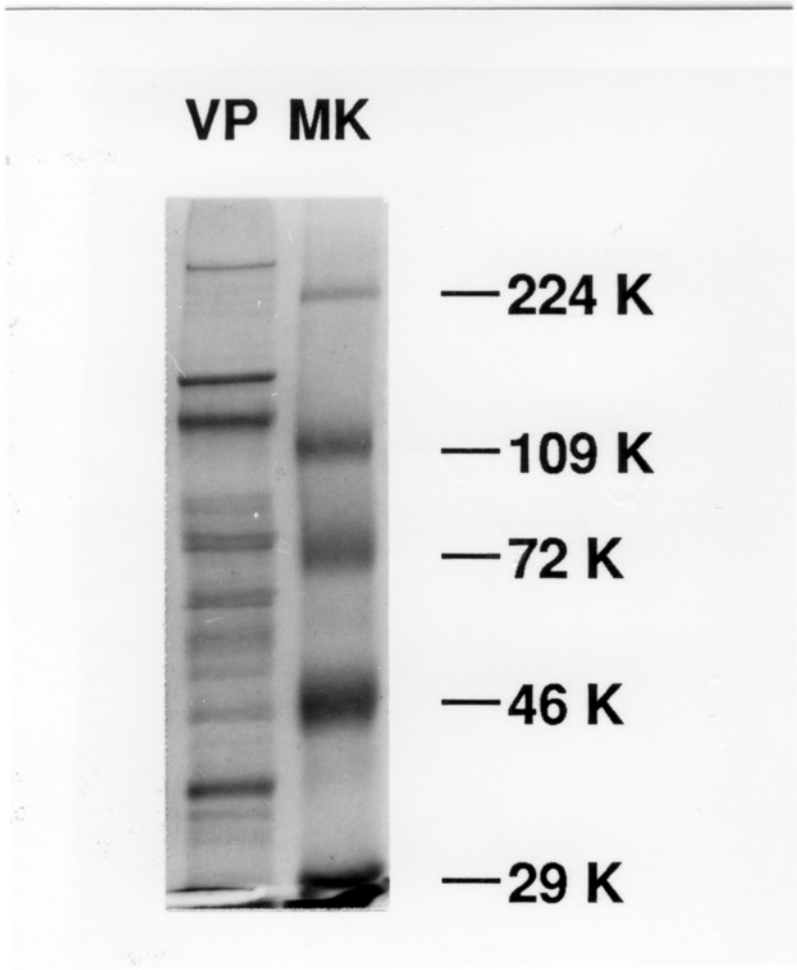

VP MK
224 K
109 K
72 K
46 K
29 K

FIGURE 22

Autoradiogram of immunoblot of HSV-1-infected cell and virion proteins probed with anti-ICP8 antiserum. Mock (M) and HSV-infected cell proteins harvested 25 HPI (25) and virion proteins (VP) were separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and probed with anti-ICP8 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the left.

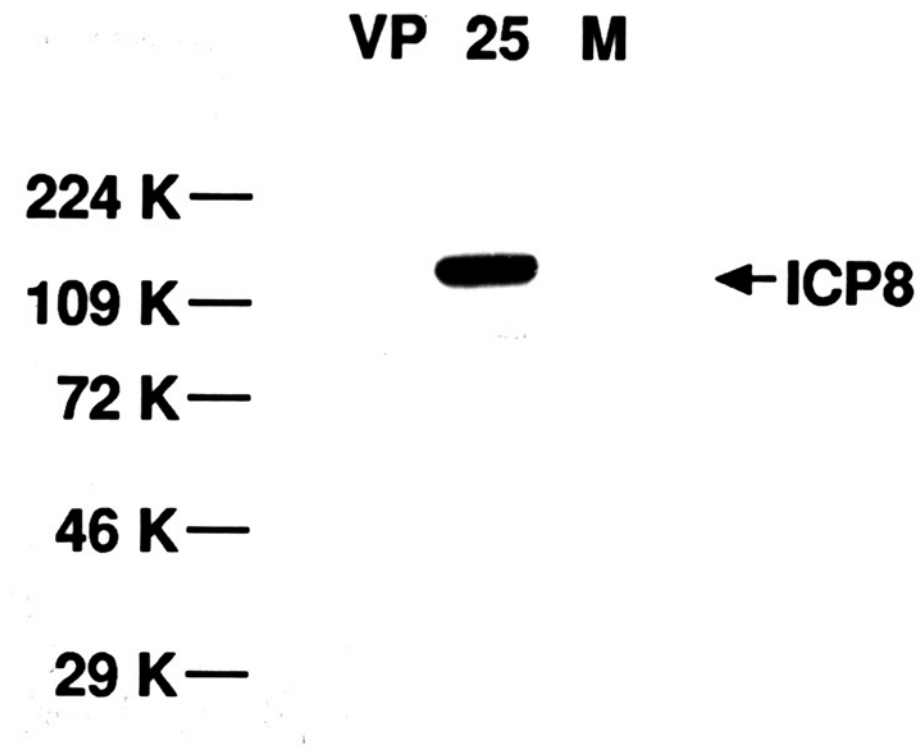
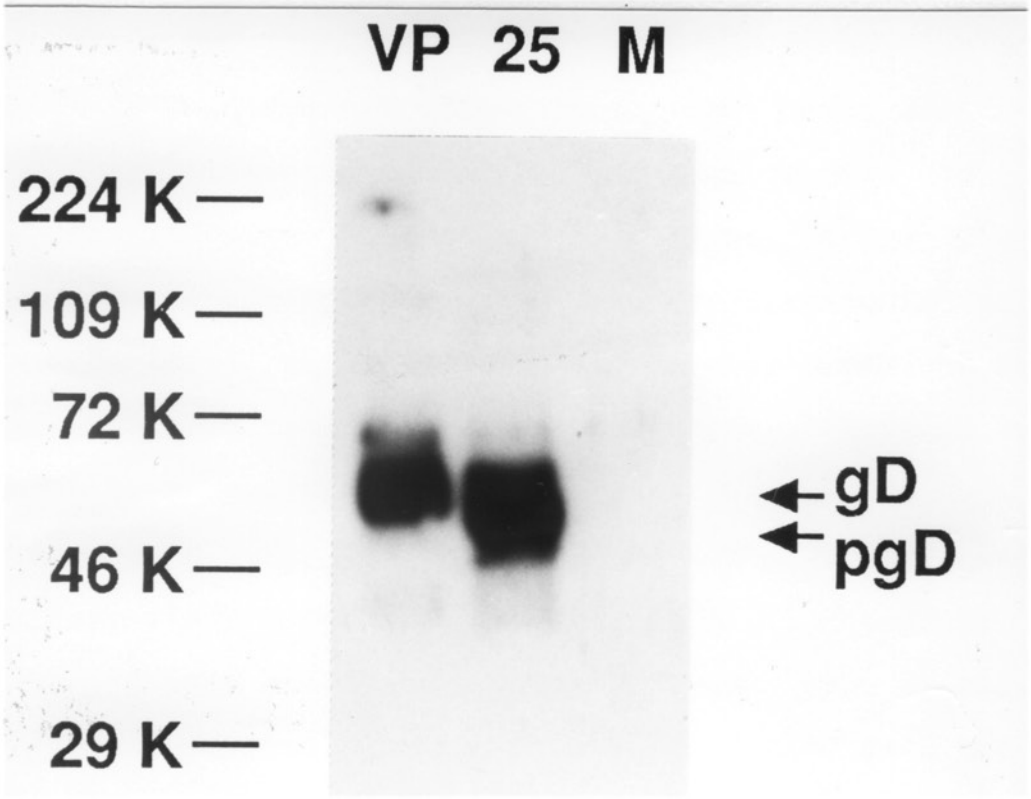

VP 25 M
224 K—
109 K—
72 K—
46 K—
29 K—
ICP8

FIGURE 23

Autoradiogram of immunoblot of HSV-1-infected cell and virion proteins probed with anti-gD antiserum. Mock (M) and HSV-infected cell proteins harvested 25 HPI (25) and virion proteins (VP) were separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and probed with polyclonal anti-gD antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the left. gD, glycoprotein D; pgD, precursor to mature gD.


VP 25 M
224 K
109 K
72 K
46 K
29 K
gD
pgD

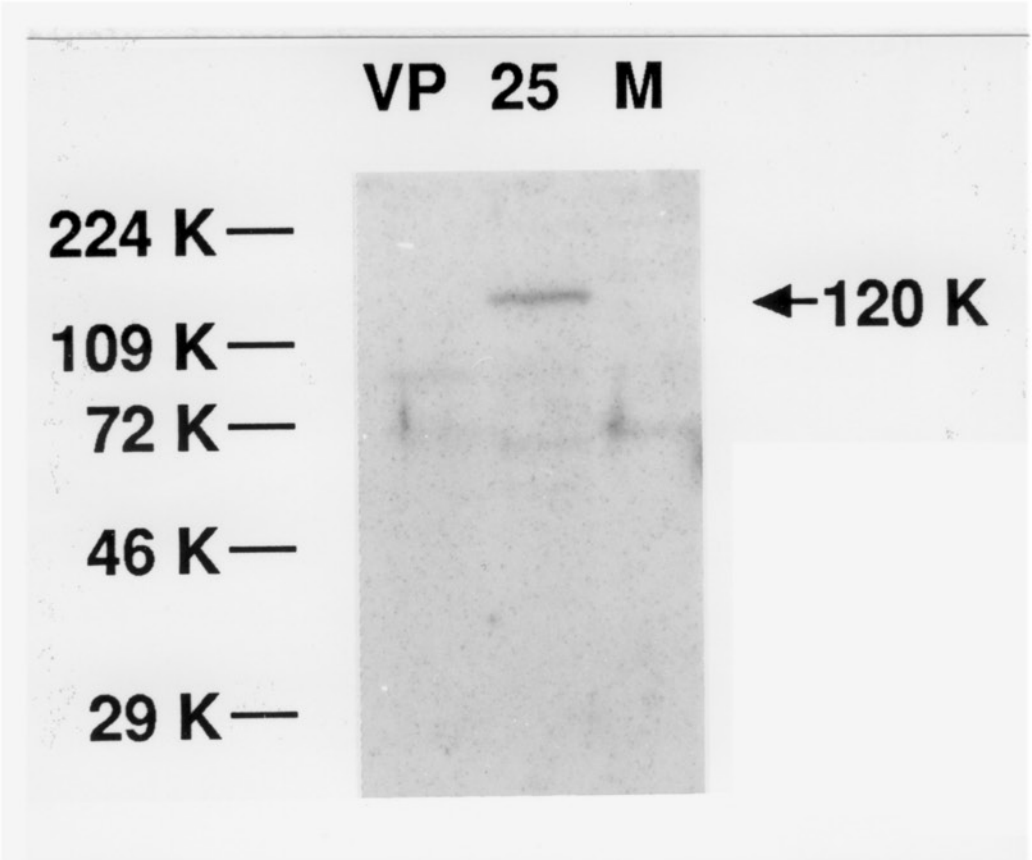
virions (Fig. 24), strongly suggesting that the UL37 protein is not a structural component of the HSV-1 virion.

Computer-assisted analysis of DNA and protein sequences

The publication of the entire DNA sequence for Epstein-Barr virus (EBV)(Baer *et al*, 1984), Varicella zoster virus (VZV)(Davison and Scott, 1986), HSV (McGeogh *et al*, 1988b), and human cytomegalovirus (HCMV)(Chee *et al*, 1990) has allowed computer-assisted analyses of these genomes and their predicted amino acid sequences. It has become apparent that the VZV genome is closely related to HSV at the genetic level, while EBV and HCMV genomes appear to be distantly related (reviewed by McGeogh, 1989). Most of the HSV genes have been found to have counterparts in VZV (Davison and McGeogh, 1986; Davison and Scott, 1986; Davison and Taylor, 1987; McGeogh *et al*, 1988) and oftentimes in both EBV (Davison and Taylor, 1987) and HCMV (Chee *et al*, 1990). The predicted homologs to the HSV-1 UL37 gene are gene 21 of VZV, BOLF1 of EBV, and HCMVUL47 of HCMV, on the basis of number of predicted amino acid residues (HSV, 1,123; VZV, 1,038; EBV, 1,239; and HCMV, 982), the predicted molecular masses (in kD) of the proteins (HSV, 120.5; VZV, 115.8; EBV, 132.7; HCMV, 110), and their genomic locations and directions of transcription in relation to surrounding genes. Using the programs Compare and Dotplot from the Genetics Computer Group Sequence Analysis Package (Devereux *et al*, 1984), the HSV-1 UL37 gene was found to have

FIGURE 24

Autoradiogram of immunoblot of HSV-1-infected cell and virion proteins probed with anti-UL37 antiserum. Mock (M) and HSV-infected cell proteins harvested 25 HPI (25) and virion proteins (VP) were separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and probed with S-1 anti-UL37 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. The molecular sizes of protein standards are indicated on the left.


VP 25 M
224 K
109 K
72 K
46 K
29 K
120 K

significant homology (47% similarity) at the amino acid level with VZV gene 21 (Fig. 25). The genetic similarity between the UL37 and VZV gene 21 proteins is shared throughout the entire length of both proteins. In contrast, the potential EBV and HCMV homologs, BOLF1 and HCMVUL47, respectively, do not share any noticeable homology at the amino acid level with either UL37 or VZV gene 21 (not shown). Analysis of the hydropathic profiles of the HSV-1 UL37 and VZV 21 proteins showed the profiles to be very similar, exhibiting a rather uniform mixture of hydrophilicity and hydrophobicity, with no obvious hydrophobic or hydrophilic domains (Fig. 26).

A search of the predicted UL37 amino acid sequence for several common protein sequence motifs demonstrated the absence of sequences corresponding to potential zinc fingers, leucine zippers, nuclear localization signals, or hydrophobic signal sequences. A potential ATP-binding site was identified between residues 452 and 490, consisting of the sequence NH_2-(β strand)-GSNVFG-(α helix)-(β strand)-COOH, which corresponds well with a predicted consensus adenine mononucleotide binding domain of NH_2-(β strand)-GXXXX-(G K α helix)-(0 to 11 amino acids)-(β strand)-COOH, reported by Bradley *et al* (1987). This ATP binding motif was not present within the VZV gene 21, EBV BOLF1, and HCMVUL47 amino acid sequences. The ability of UL37 to bind ATP and hydrolyze ATP was not pursued in this study.

FIGURE 25

Dotplot comparison of the predicted amino acid sequences of HSV-1 UL37 and VZV gene 21. Homology search was performed using a window of 19 and stringency of 13 using the Dotplot program of the Genetics Computer Group Sequence Analysis Package (Madison, Wisc).

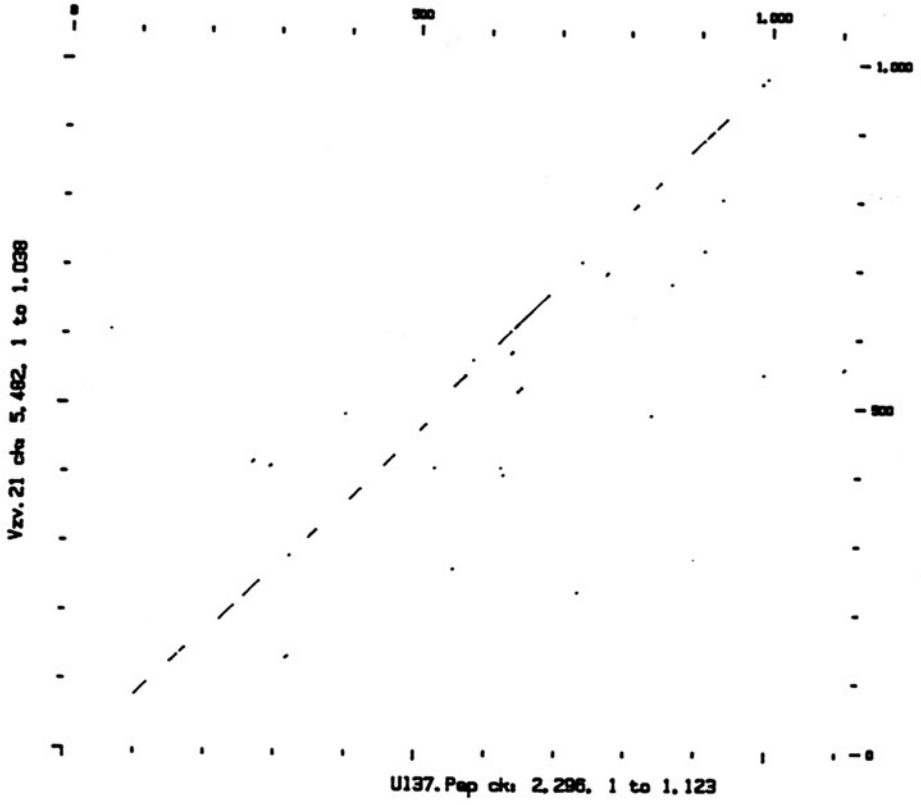
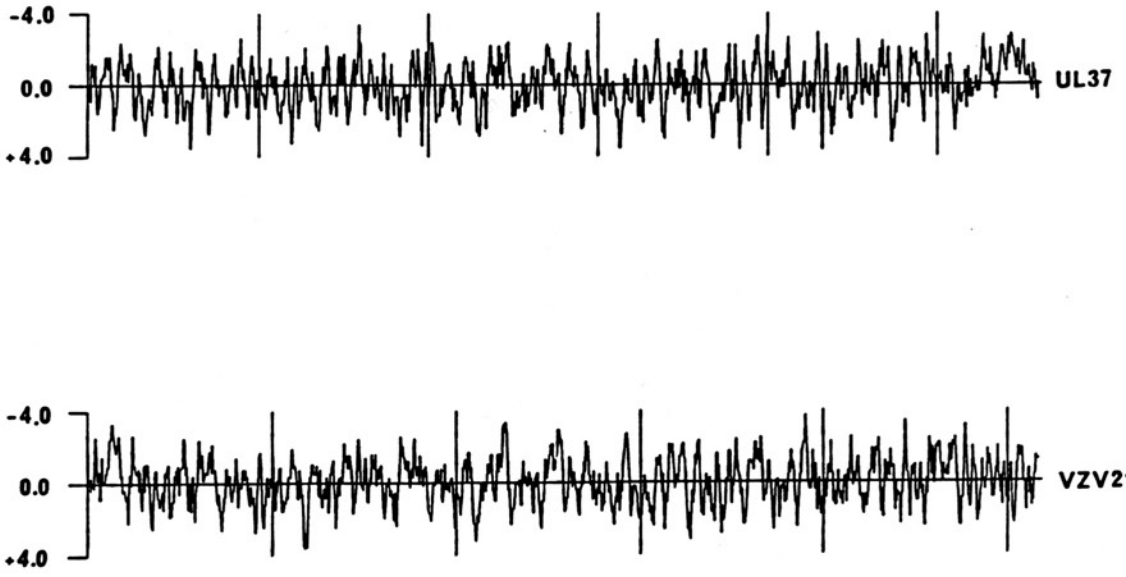

500
1,000
1,000
500
0
0
Vzv.21 cks 5,482. 1 to 1,038
U137.Pep cks 2,298. 1 to 1,123

FIGURE 26

Hydropathy plots of predicted amino acid sequences of HSV-1 UL37 and VZV gene 21. Hydropathic profiles were generated by the Protolyze program from Scientific and Educational Software (Stateline, Pa.). Hydrophilic regions are located above the zero line, and hydropathic regions are located below the zero line.


-4.0
0.0
+4.0
UL37
-4.0
0.0
+4.0
VZV21

III. Biochemical studies of the UL37 protein

Single-stranded & double-stranded DNA column chromatography using HSV-infected cell proteins

Since the UL37 gene product does not appear to be an HSV-1 structural protein, other reasonable possibilities for its function would be involvement in either the later phases of viral DNA replication or viral gene expression. Both of these possibilities might require the UL37 protein to interact with nucleic acids. To determine whether the UL37 protein could interact directly with DNA in a nonspecific (sequence-independent) manner, the binding ability of the UL37 protein to both single-stranded (SS) and double-stranded (DS) DNA columns was assessed.

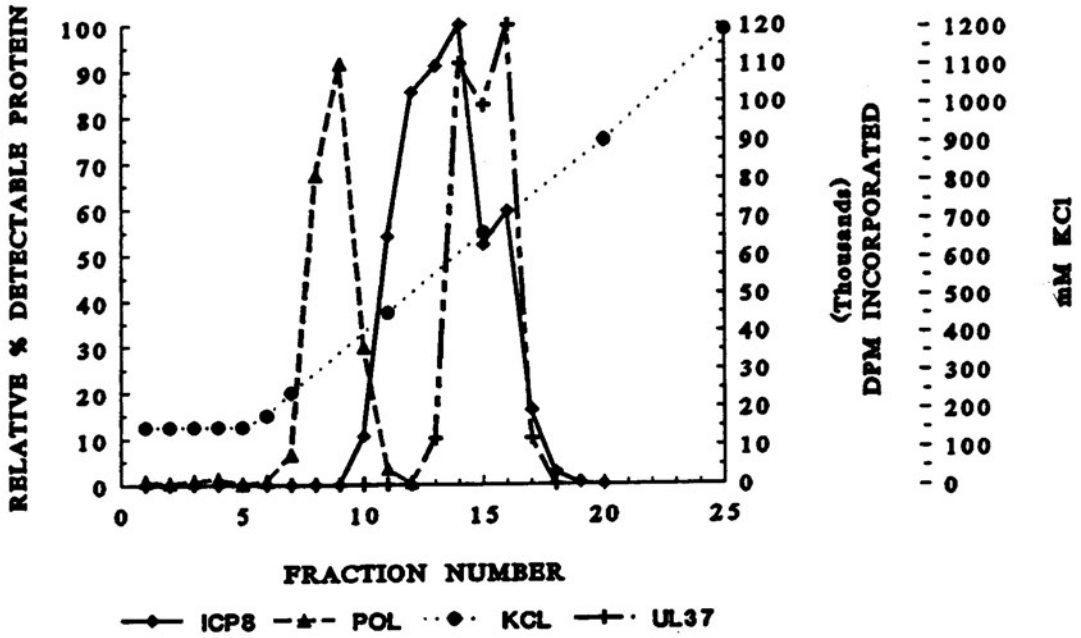
To determine if the UL37 protein was capable of binding single-stranded DNA (SS DNA), proteins from HSV-1 infected Vero or CV-1 cells were harvested at 24 HPI and loaded onto either a 10- or 30-ml SS DNA agarose column. Bound proteins were eluted with a linear 150 mM to 1.5 M KCl gradient (in some experiments 100 mM to 1 M KCl gradient). Fractions were collected and analyzed by immunoblotting using 487 antiserum. Routinely, as a control for these experiments, each fraction was also analyzed for the presence of ICP8 by immunoblot using ICP8-specific antiserum. In one experiment, as an additional control, DNA polymerase activity was measured in each fraction by Dr. William Ruyechan's laboratory. Autoradiograms of immunoblots using

487 and ICP8 antisera were densitometrically scanned, and the results from one experiment including DNA polymerase activities of each fraction are graphically represented in Fig. 27. The HSV DNA polymerase activity eluted between 300 and 400 mM KCl, while both the UL37 and ICP8 proteins exhibited a stronger affinity for SS DNA, eluting between 600 and 700 mM KCl. The elution profiles of HSV DNA polymerase activity and ICP8 protein from this experiment agree with previously published reports from several laboratories (Bayliss *et al*, 1975; Powell and Purifoy, 1976; Purifoy and Powell, 1976; Powell and Purifoy, 1977; Knipe *et al*, 1982; Ruyechan and Weir, 1984). This was the first evidence that the UL37 protein was capable of eluting from SS DNA columns. The finding that UL37 eluted from these columns at such a high salt concentration, co-eluting with ICP8, the HSV major DNA binding protein, was quite significant. Since the UL37 and ICP8 proteins migrated to the same position on SDS-PAGE, requiring an immunoblot using specific antisera to distinguish between the two proteins, previous investigators, using radiolabelled HSV proteins, failed to detect UL37 among the HSV DNA binding proteins eluted from such columns.

In all SS DNA column chromatography experiments using HSV-infected cell extracts, the UL37 protein co-eluted with the ICP8 protein with salt concentrations greater than 300 mM KCl and generally peaked within the range of 400 to 700

FIGURE 27

Graph of elution profiles of HSV-1 UL37, ICP8, and DNA polymerase proteins from SS DNA-agarose column chromatography. HSV-1 infected cell proteins were harvested at 24 HPI from Vero cells as described in Materials and Methods and loaded onto a 30-ml SS DNA agarose column. The column was washed and bound proteins eluted using a linear KCl gradient (second scale on the right). DNA polymerase activity was measured as described in Materials and Methods with ^{3}H-dTTP incorporation (first scale on right). Proteins in each fraction were separated on SDS-9% polyacrylamide gels and immunoblots probed with anti-ICP8 or 487 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. Autoradiograms of immunoblots were scanned densitometrically and data summarized in this graph. The values shown represent the relative abundance of each protein compared to its peak level detected (scale on the left).

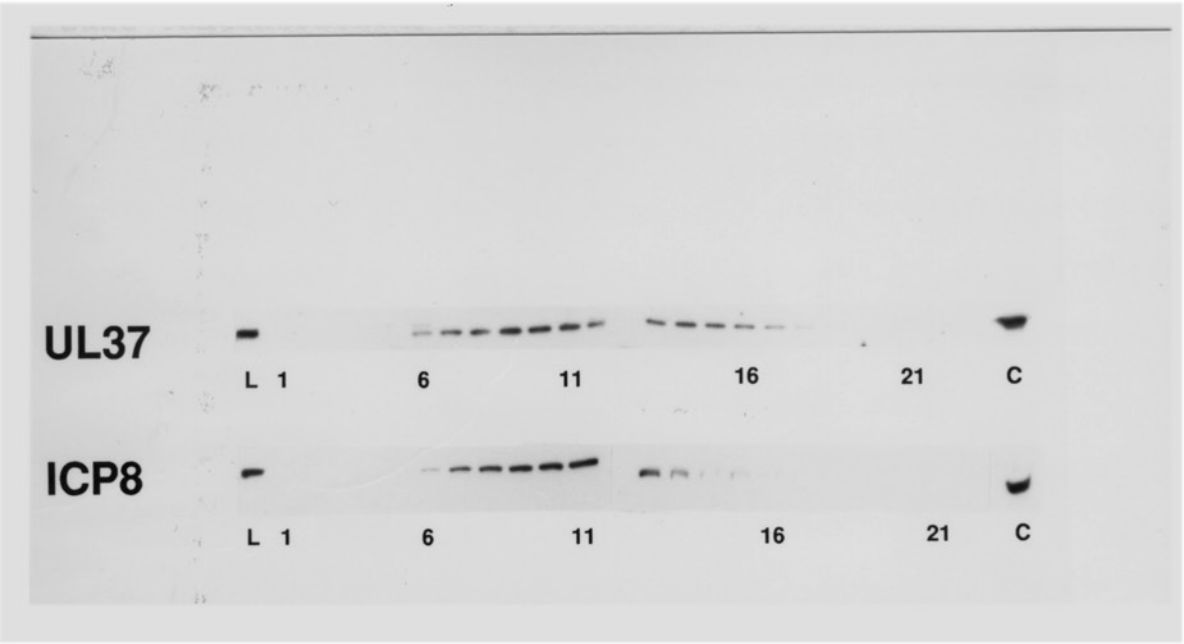

RELATIVE % DETECTABLE PROTEIN
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
120
110
100
90
80
70
60
50
40
30
20
10
0
(Thousands)
DPM INCORPORATED
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
mM KCl
FRACTION NUMBER
ICP8
POL
KCL
UL37

mM KCl. To determine whether the high affinity binding ability of these proteins was diminished upon repeated exposure to SS DNA, the pooled fractions containing the peak of eluted UL37 and ICP8 proteins were re-loaded onto a second SS DNA column and bound proteins eluted and analyzed for UL37 and ICP8 proteins. Immunoblots probed with UL37 and ICP8 antisera demonstrated a co-elution of UL37 and ICP8 in fractions ranging from 375 to 750 mM KCl with both proteins peaking at 500 mM KCl (Fig. 28). These results were identical to the results of all other SS DNA column chromatography performed using HSV-infected cell proteins.

Since the UL37 protein exhibited a high affinity binding to SS DNA, similar to that of ICP8, and ICP8 was previously reported to bind double-stranded (DS) DNA (Bayliss *et al*, 1975; Purifoy and Powell, 1976; Powell and Purifoy, 1977; Powell *et al*, 1981; Ruyechan and Weir, 1984), UL37 binding to DS DNA was investigated to determine if indeed UL37 could bind DS DNA or if UL37 could be separated from ICP8. Proteins from HSV-infected CV-1 cells were harvested at 24 HPI, loaded onto a 10-ml DS DNA cellulose column, and bound proteins eluted with a linear 100 mM KCl to 1 M KCl gradient. Proteins in each fraction were probed by immunoblot for UL37, ICP8, and UL42 proteins using specific antisera. The presence of UL42, a known DS DNA binding protein (Marsden *et al*, 1987), served as an additional control. The UL37 protein demonstrated binding

FIGURE 28

Autoradiograms of immunoblots of HSV-1-infected cell proteins eluted from a SS DNA-agarose column probed with 487 and anti-ICP8 antisera. HSV-1-infected cell proteins were harvested at 24 HPI from CV-1 cells as described in Materials and Methods and loaded onto a 10-ml SS DNA-agarose column. The column was washed and bound proteins eluted using a linear KCl gradient. Proteins in each fraction were separated on SDS-9% polyacrylamide gels and immunoblots probed with 487 or anti-ICP8 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. A sample of the load (L) and numbered fractions are shown. The control lane (C) contains HSV-1-infected cell proteins harvested at 24HPI.


UL37
L 1 6 11 16 21 C
ICP8
L 1 6 11 16 21 C

to DS DNA and co-eluted with the ICP8 protein (Fig. 29), with the majority of each protein eluting between 250 and 500 mM KCl, peaking between 300 and 400 mM KCl (Fig. 30). The UL42 protein eluted from DS DNA over a broad range of salt concentrations as previously reported (Marsden *et al*, 1987).

Additionally, the use of other affinity chromatography substrates such as heparin sepharose and phosphocellulose which are routinely used to purify HSV DNA binding proteins did not result in the separation of UL37 and ICP8 proteins (data not shown).

Single-stranded DNA column chromatography using V37-infected cell proteins

The finding that the UL37 protein bound SS and DS DNA columns indicated that either UL37 bound these DNA substrates directly or interacted with other proteins which themselves bound DNA. The observation that the UL37 protein consistently co-eluted with the ICP8 protein raised the possibility that UL37 interacted with the ICP8 protein in binding DNA. To determine whether the UL37 protein was capable of binding SS DNA in the absence of other HSV proteins, the DNA binding ability of UL37 protein expressed by the vaccinia recombinant virus, V37, was assessed. CV-1 cells were infected with V37 at an MOI of 5, and infected cell proteins harvested between 17 and 27 HPI. The V37 extract was loaded onto a 10-ml SS DNA agarose column and

FIGURE 29

Autoradiograms of immunoblots of HSV-1-infected cell proteins eluted from a DS DNA-cellulose column probed with 487 and anti-ICP8 antisera. HSV-1-infected cell proteins were harvested at 24 HPI from CV-1 cells as described in Materials and Methods and loaded onto a 10-ml DS DNA-cellulose column. The column was washed and bound proteins eluted using a linear KCl gradient. Proteins in each fraction were separated on SDS-9% polyacrylamide gels and immunoblots probed with 487, anti-UL42 (not shown), or anti-ICP8 antiserum. Antibody binding was detected with ^{125}I-labelled protein A. Numbered fractions containing detected protein are shown.

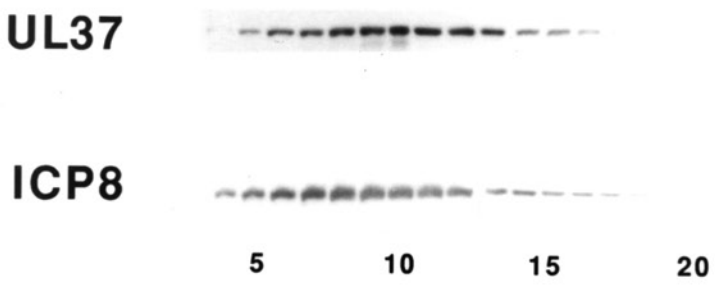
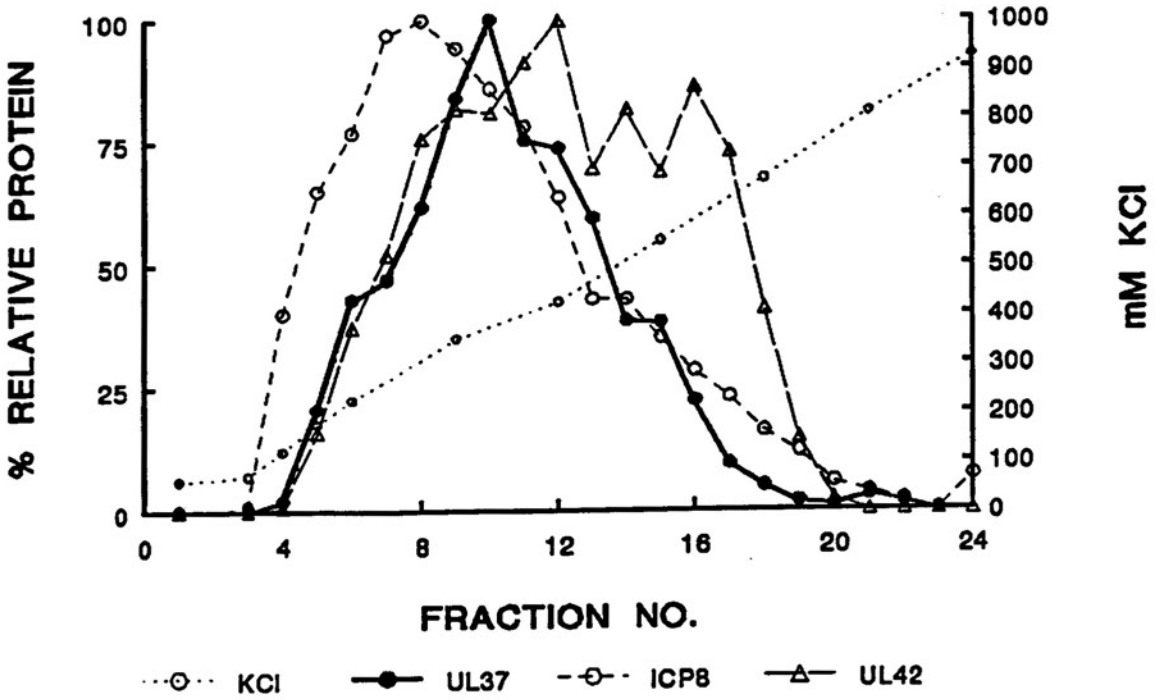

UL37
ICP8
5
10
15
20

FIGURE 30

Graph of elution profiles of HSV-1 UL37, ICP8, and UL42 proteins from DS DNA-cellulose column chromatography. Autoradiograms of immunoblots described in Fig. 29 were scanned densitometrically and data summarized in this graph. The values shown represent the relative abundance of each protein compared to its peak level detected (scale on the left). KCl elution gradient is included (scale on the right).


% RELATIVE PROTEIN
100
75
50
25
0
0
4
8
12
16
20
24
FRACTION NO.
1000
900
800
700
600
500
400
300
200
100
0
mM KCl
KCl
UL37
ICP8
UL42

bound proteins eluted by a linear 50 mM to 1.0 M KCl gradient. Fractions were collected and probed with anti-1.1 antiserum on immunoblot. The vaccinia-expressed UL37 protein (V37 protein) was detected within the column wash and was absent within the gradient elution (Fig. 31, line A), demonstrating the inability of UL37 to bind DNA in the absence of ICP8 and other HSV proteins.

Isoelectric focusing of HSV and V37-infected cell proteins

Since the separation of UL37 and ICP8 proteins using several affinity chromatography techniques routinely used for ICP8 purification was unsuccessful, isoelectric focusing of HSV-infected cell proteins was performed, taking advantage of the distinct theoretical isoelectric points of these two proteins (UL37 pI = 6.04, ICP8 pI = 6.48). CV-1 cells were infected with HSV at an MOI of 5 and harvested at 24 HPI, prepared essentially as for SS DNA column chromatography except without salt (See Solubility section below). Preparative isoelectric focusing was performed using a Bio-Rad Rotofor cell under reducing and nondenaturing conditions. pH determinations on the harvested fractions indicated a linear pH gradient, and immunoblot analysis was performed using antisera specific for UL37 and ICP8. UL37 and ICP8 bands were visualized in a broad range, co-fractionating at pH 4.92 to 7.66 (Fig. 32, fractions 6 to 14) and peaking at pH 5.83. Separation of the UL37 and ICP8 proteins by this technique was

FIGURE 31

Immunoblot analysis of V37 and d21-infected cell proteins from SS DNA column chromatography experiments.

(A) V37-infected cell proteins were harvested 27 HPI and loaded onto a 10-ml SS DNA-agarose column. Column chromatography was performed as described with fractions collected continuously, starting at the time of loading. Proteins in each fraction were separated on SDS-9% polyacrylamide gels and immunoblots probed with 1.1 antiserum. Antibody binding was detected with ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. A sample of the load (L), flow-through (FT), and initial portion of the wash (W1-W6) are shown.

(B) d21-infected cell proteins were harvested 24 HPI and loaded onto a 10-ml SS DNA-agarose column. Column chromatography was performed as described with fractions collected continuously, starting at the time of loading. Proteins in each fraction were separated on SDS-9% polyacrylamide gels and immunoblots probed with 1.1 antiserum. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. A sample of the load (L), flow-through (FT), and initial portion of the wash (W1-W5), and first fraction of the gradient (F1) are shown.

A.

B.

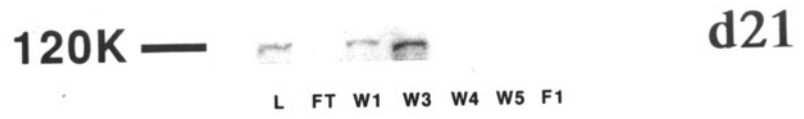
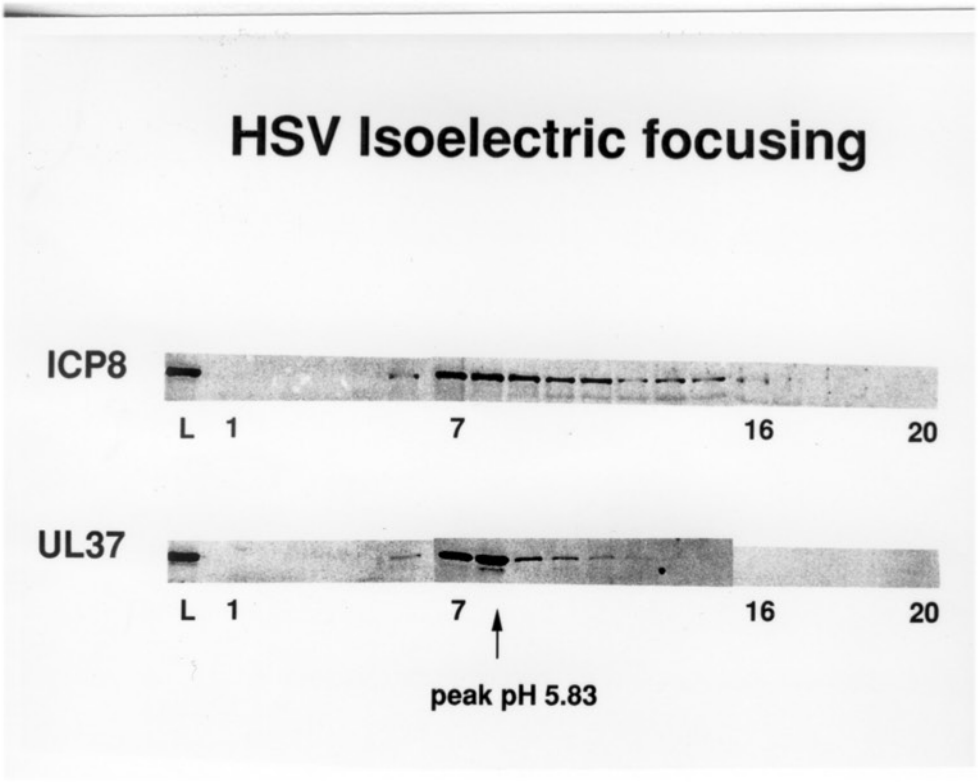


FIGURE 32

Immunoblot analysis of HSV-1-infected cell proteins harvested from isoelectric focusing cell probed with anti-ICP8 and 1.1 antisera. HSV-1-infected cell proteins harvested 24 HPI were diluted and loaded into a Rotofor cell. Isoelectric focusing was performed as described. Proteins in each fraction were separated on SDS-9% polyacrylamide gels, and immunoblots including a sample of the diluted load (L) and harvested fractions (1-20) probed with anti-ICP8 (C-terminal peptide) and 1.1 antiserum are shown. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager.


HSV Isoelectric focusing
ICP8
L 1 7 16 20
UL37
L 1 7 16 20
peak pH 5.83

unsuccessful, and the presence of both proteins over such a broad range potentially indicated differing degrees of interaction between these proteins and/or other HSV proteins or the existence of different isoforms of each protein, especially ICP8. Quantitation of band intensity using the Molecular Dynamics Phosphorimager in Fig. 33 demonstrated a co-fractionation of the two proteins as well as a "shoulder" of ICP8 protein, distinct from UL37, at a higher pH (pH 6.5 to 7.5). The co-fractionation and peak at a lower pH than predicted for both proteins and the distinct ICP8 shoulder were observed in repeat experiments.

As a control experiment, isoelectric focusing of V37-infected cell proteins was performed to determine if the UL37 in the absence of ICP8 and other HSV proteins would again focus in a broad range at a lower pH or at its predicted pI. V37-infected cell proteins were prepared and separated by preparative isoelectric focusing as described above. Immunoblot analysis of the harvested fractions using anti-1.1 antiserum demonstrated the V37 protein focusing very tightly as a peak at pH 5.96 (Fig. 34), consistent with the predicted pI of 6.0.

Solubility of HSV and V37-infected cell proteins

Since the HSV-infected cell protein preparations (the soluble fraction) used in SS DNA column chromatography were prepared at 150 mM KCl and the presence of little or no salt was recommended for isoelectric focusing experiments,

FIGURE 33

Graph of fractionation profile of UL37 and ICP8 proteins by isoelectric focusing of HSV-1-infected cell proteins. Quantitation of band intensity in Fig. 32 using the Molecular Dynamics Phosphorimager was performed and data summarized in this graph. The values shown represent the relative abundance of each protein compared to its peak level detected.

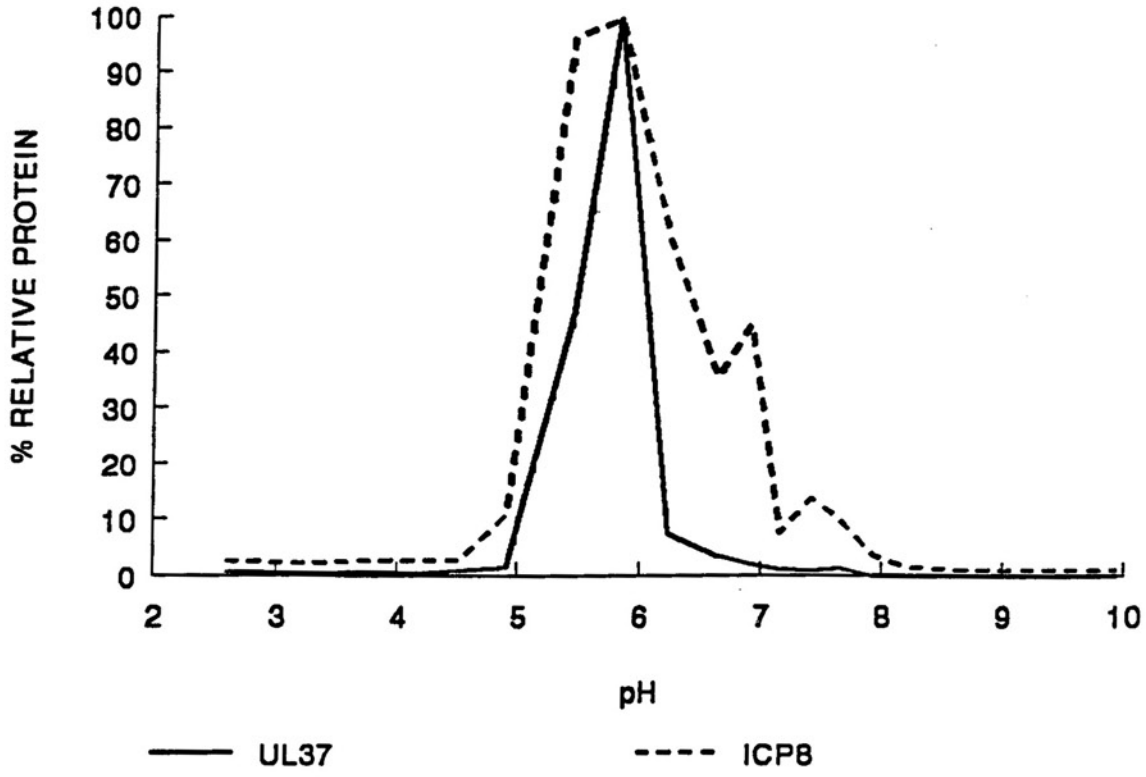
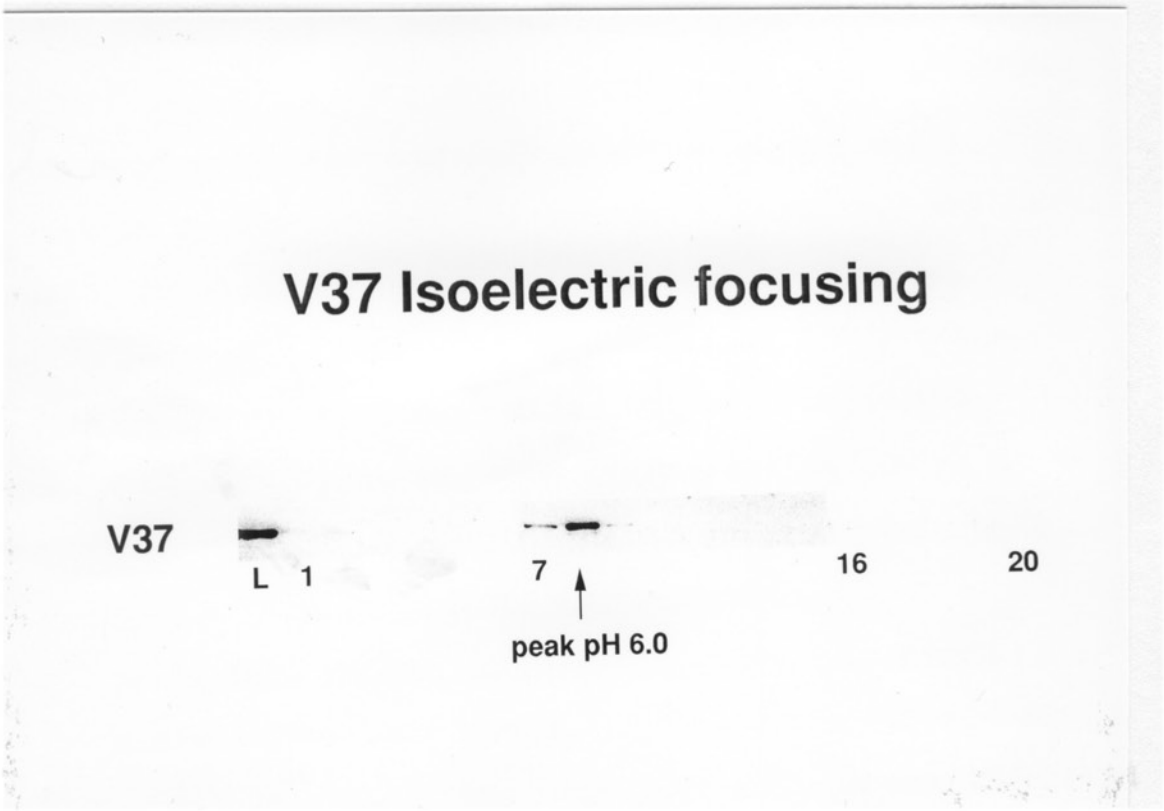

% RELATIVE PROTEIN
100
90
80
70
60
50
40
30
20
10
0
2
3
4
5
6
7
8
9
10
pH
UL37
ICP8

FIGURE 34

Immunoblot analysis of V37-infected cell proteins harvested from isoelectric focusing cell probed with anti-1.1 antiserum. V37-infected cell proteins harvested 24 HPI were diluted and loaded into a Rotofor cell. Isoelectric focusing was performed as described. Proteins in each fraction were separated on SDS-9% polyacrylamide gels, and immunoblots including a sample of the diluted load (L) and harvested fractions (1-20) probed with 1.1 antiserum are shown. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager.


V37 Isoelectric focusing
V37
L
1
7
16
20
peak pH 6.0

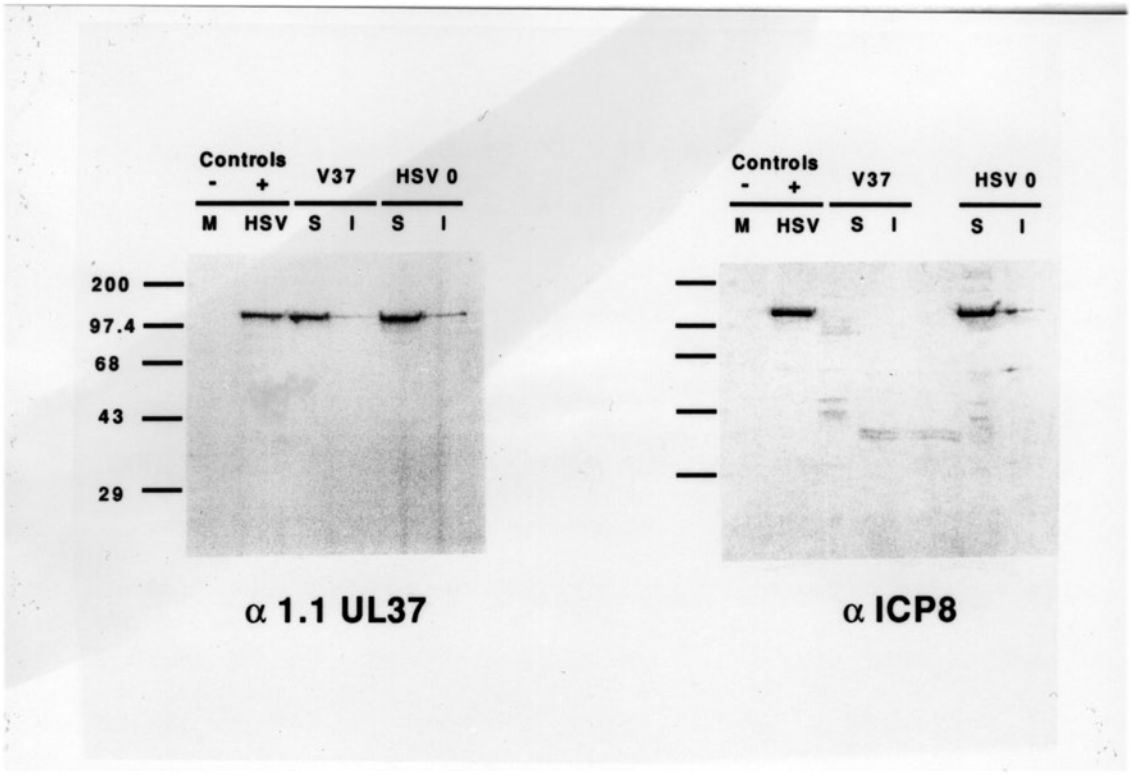
soluble and insoluble infected cell proteins were analyzed for UL37 content by immunoblot using anti-1.1 UL37 antiserum to determine if the UL37 protein present within isoelectric focusing preparations was representative of the total UL37 protein present within infected cells. The soluble HSV-infected cell proteins prepared with no salt contained the majority of both UL37 and ICP8 proteins with a small proportion present within the insoluble fraction, indicating adequate solubility of UL37 for isoelectric focusing preparations (Fig. 35). The solubility of V37 protein without salt was not tested since V37 protein showed adequate solubility at salt concentrations as low as 50 mM KCl.

SS DNA column chromatography
using ICP8 mutant (d21)-infected cell proteins

The possiblity that the UL37 protein was capable of forming a stable complex with the ICP8 protein was indicated by the following observations: (1) the UL37 and ICP8 proteins from HSV-infected cells co-eluted from both SS and DS DNA columns, (2) the V37 protein, in the absence of other HSV proteins, particularly ICP8, failed to bind DNA, and (3) all attempts at separation of the UL37 and ICP8 proteins using a variety of chromatographic techniques including isoelectric focusing were unsuccessful. To investigate whether ICP8 was required for UL37 to bind DNA, an ICP8 deletion mutant, d21, was obtained from Dr. Priscilla

FIGURE 35

Immunoblot analysis of soluble and insoluble fractions of HSV and V37-infected cell protein preparations. Insoluble (I) and soluble (S) proteins prepared from HSV-1-infected cells at 24 HPI without salt (HSV 0) and V37-infected cells with 50 mM KCl (V37) to be used in isoelectric focusing and SS DNA column chromatography experiments were separated on SDS-9% polyacrylamide gels. Immunoblots probed with 1.1 and anti-ICP8 (C-terminal peptide) antiserum are shown. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. Control lanes ([-] and [+]) include infected cell proteins from mock (M) and HSV-1-infected cells (HSV), respectively.


Controls
- +
V37
HSV 0
M HSV S I S I
200
97.4
68
43
29
α 1.1 UL37
Controls
- +
V37
HSV 0
M HSV S I S I
α ICP8

Schaffer for SS DNA column chromatography.

The d21 virus is an HSV recombinant isolated in an ICP8 complementing cell line, U-47, which contains three copies of the ICP8 gene per haploid genome (Orberg and Schaffer, 1987). d21 has 260 amino acids deleted from the ICP8 gene including the entire zinc binding domain (Gao *et al*, 1988; Gupte *et al*, 1991) and 23 amino acids into what has been identified as a "DNA binding region" (Leinbach and Heath, 1988; Gao and Knipe, 1989; Wang and Hall, 1990; Gao and Knipe, 1991)(Fig. 36). When d21 is grown in noncomplementing cells, this mutation results in an HSV DNA negative phenotype, and the ICP8 molecule remains in the cytoplasm (Orberg and Schaffer, 1987). Since a functional ICP8 molecule is essential for HSV DNA replication (Conley *et al*, 1981; Powell *et al*, 1981; Weller *et al*, 1983; Challberg, 1986; Wu *et al*, 1988; McGeogh *et al*, 1988a), and UL37 demonstrates γ1 expression, it was presumed that d21 infection of noncomplementing cells would result in reduced UL37 expression, similar to that of PAA-treated cells. To determine whether detectable levels of UL37 would be generated from such an infection, d21-infected cell proteins were prepared by infecting noncomplementing CV-1 cells and complementing U-47 cells and analyzed by immunoblot using antisera specific for the UL37 and ICP8 proteins. As shown in Fig. 37, the UL37 protein was detected in both CV-1 and U-47 cells infected with d21 with a diminished level of

FIGURE 36

Schematic showing deleted portion in the d21 virus relative to the functional domains identified for ICP8. The functional domains of ICP8 previously described (Introduction, Functional domains of ICP8, and Fig. 8) are represented as well as the 780 bp (260 aa) in frame deletion in d21.

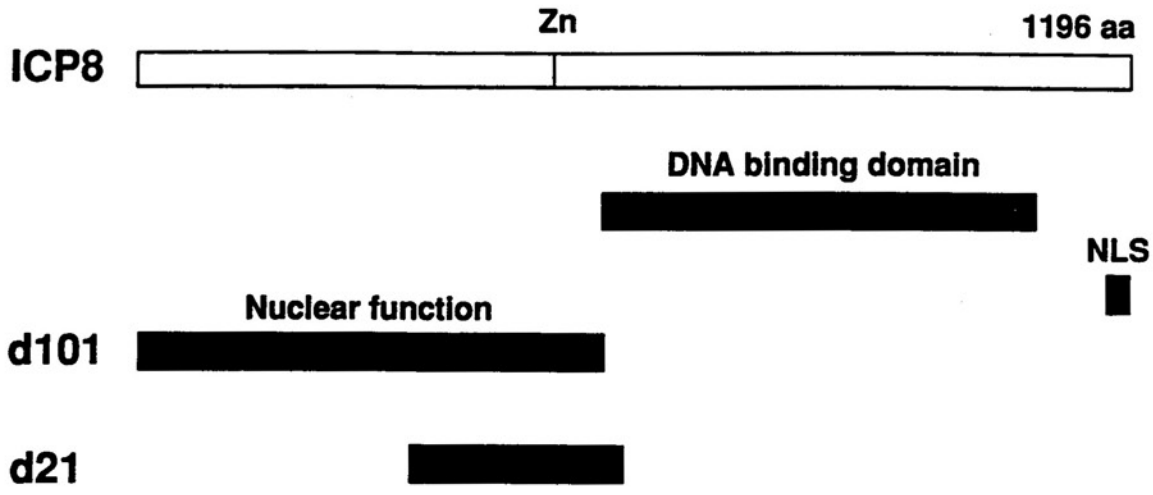
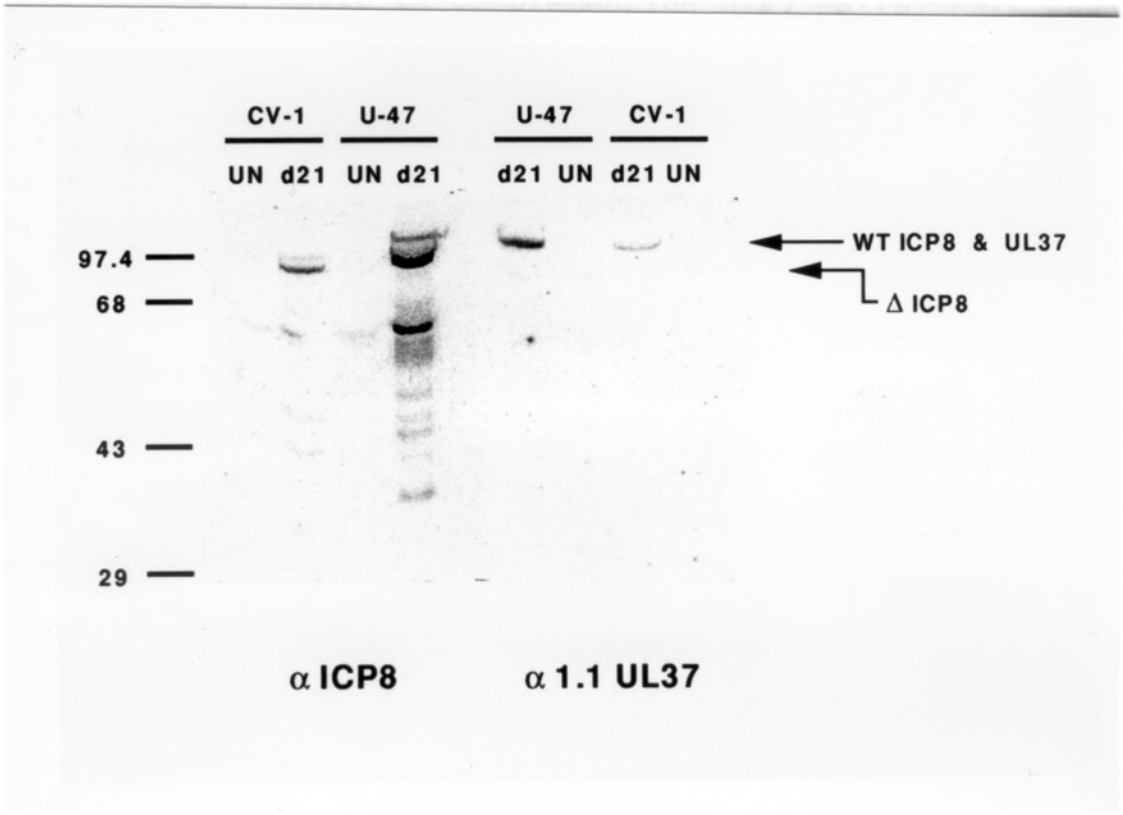

Zn
1196 aa
ICP8
DNA binding domain
NLS
Nuclear function
d101
d21

FIGURE 37

Immunoblot analysis of d21-infected cell proteins from complementing (U-47) and noncomplementing (CV-1) cells. Uninfected (UN) and d21-infected cell proteins (d21) harvested 24 HPI from CV-1 and U-47 cells were separated on SDS-9% polyacrylamide gels. Immunoblots probed with 1.1 and ICP8 (C-terminal peptide) antisera are shown. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. Molecular sizes of protein standards are indicated on the left.


CV-1
U-47
U-47
CV-1
UN d21 UN d21
d21 UN d21 UN
97.4
68
43
29
WT ICP8 & UL37
Δ ICP8
α ICP8
α 1.1 UL37

expression found in the noncomplementing cells. In the noncomplementing cells, only the deleted ICP8 protein expressed by the the d21 mutant was detected, approximately 85 kD in size, while in complementing U-47 cells, both the deleted ICP8 protein expressed by the d21 mutant and the wild-type ICP8 protein expressed by the cell line were observed.

To determine whether the UL37 protein could bind DNA in the absence of functional ICP8 protein, the extracts from d21-infected CV-1 cells were used as a source of HSV UL37 protein and defective ICP8. The d21-infected cell proteins were harvested from CV-1 cells infected at an MOI of 5 and loaded onto a 5-ml SS DNA agarose column. The column was washed to remove unbound proteins and then bound proteins eluted with a linear KCl gradient as described previously. Immunoblot analysis of the fractions collected indicated the presence of the UL37 protein only in the column wash and not within the elution gradient, indicating the inability of UL37 to bind directly to DNA (Fig. 31, line B). The deleted ICP8 protein was also unable to bind SS DNA and was detected in the column wash (data not shown). These results show that, in d21-infected cells as compared to HSV-infected cells, in the absence of functional ICP8 binding, the binding of the UL37 protein to DNA is no longer observed, indicating that an ICP8-UL37 protein interaction is responsible for the binding of UL37 to DNA which is observed

with HSV-infected cell proteins.

Clearly, the deleted ICP8 protein of the d21 virus was incapable of binding DNA. Whether this deletion had an additional effect on the ability of the deleted ICP8 protein to complex with UL37 was addressed by immunoprecipitation studies (see Nuclear localization studies, below).

Allen Albright, a graduate student in our laboratory, has begun investigation of the interaction between the UL37 and ICP8 proteins. His initial studies have demonstrated that the UL37 and ICP8 proteins from HSV-infected cells can be co-precipitated using an antiserum directed against the ICP8 protein (personal communication). The results demonstrating an ICP8-UL37 interaction reported in this study were entirely consistent with this finding.

Nuclear localization studies
using HSV, V37, & d21-infected cell proteins

Having demonstrated that the ICP8-UL37 protein interaction was responsible for the binding of UL37 to DNA, it was hypothesized that the ICP8-UL37 complex may result in a modified function of ICP8 late in viral replication, most likely in the nucleus. The nuclear localization of ICP8 has been extensively studied by immunofluorescence studies including confocal microscopy showing changes in intra-nuclear distribution coinciding with viral DNA replication (Quinlan *et al*, 1984; de Bruyn Kops and Knipe, 1988). A nuclear localization signal located at the C-terminus of the

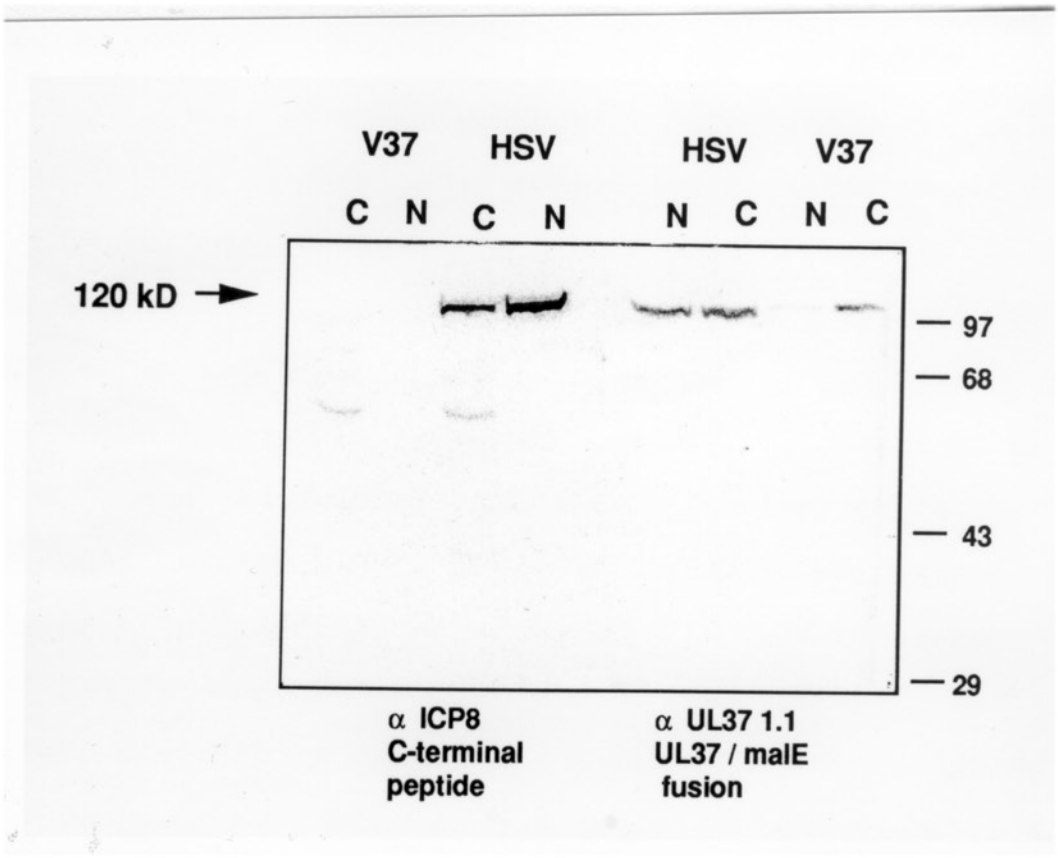
ICP8 protein has been identified as sufficient for nuclear localization (Gao and Knipe, 1989).

Since the UL37 protein has no nuclear localization signal yet clearly demonstrates an ability to bind DNA indirectly by an interaction with ICP8, it was hypothesized that ICP8 may associate with the UL37 protein in the cytoplasm and facilitate its transport to the nucleus. Since adequate immunologic reagents for immunofluorescence studies with HSV-infected cells (monoclonal antibodies or polyclonal antibodies with a low nonspecific background) were unavailable, cell fractionation studies were performed to determine whether the UL37 protein was transported to the nucleus in the presence and absence of ICP8 protein. Nuclear and cytoplasmic fractions were prepared from HSV and V37-infected cells at 24 HPI (as described in Materials and Methods), and their proteins were separated by SDS-PAGE, and analyzed for UL37 and ICP8 content by immunoblotting with specific antisera. Among HSV-infected cell proteins, both UL37 and ICP8 were detected in both cytoplasm and nucleus (Fig. 38), demonstrating the nuclear localization of UL37 during HSV infection. In contrast, the V37 protein remained cytoplasmic in the absence of ICP8 and other HSV proteins. In this experiment, a slight band was detected in the nuclear fraction from V37-infected cells which may indicate a small degree of contamination with cytoplasmic proteins.

To determine whether the ICP8 protein was responsible

FIGURE 38

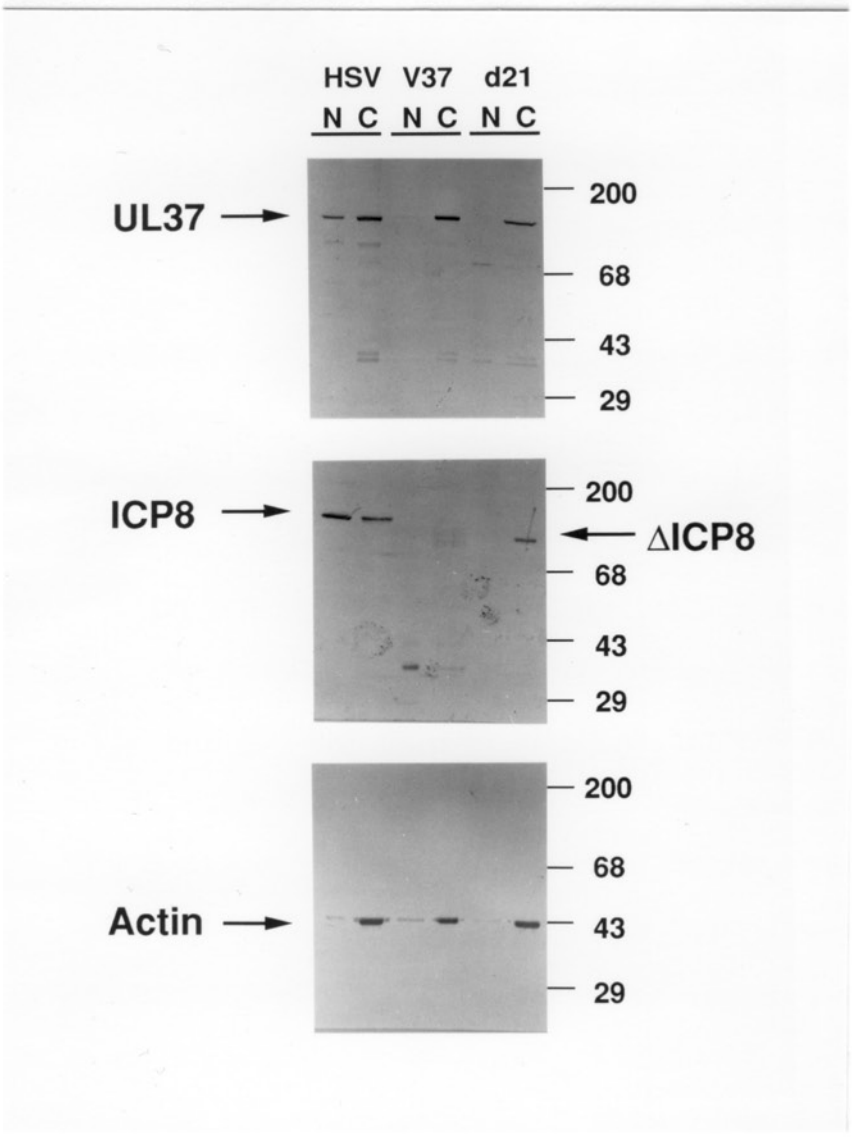
Immunoblot analysis of cytoplasmic and nuclear fractions of HSV-1 and V37-infected cells. Cytoplasmic (C) and nuclear (N) fractions were prepared from HSV-1-infected cells and V37-infected cells harvested 24 HPI as described in Materials and Methods. Proteins were separated on a SDS-9% acrylamide gel and immunoblots probed with 1.1 and ICP8 (C-terminal peptide) antisera. Antibody binding was detected by ^{125}I-labelled protein A followed by image analysis on an Image Quant Phosphorimager. Molecular sizes of protein standards are indicated on the right.


V37
HSV
HSV
V37
C N C N N C N C
120 kD
97
68
43
29
α ICP8
C-terminal
peptide
α UL37 1.1
UL37 / malE
fusion

for the nuclear localization of the UL37 protein, cell fractionation of d21-infected CV-1 cells was performed for immunoblot analysis. Although d21 contains the C-terminal nuclear localization signal, the deletion in the d21 ICP8 protein inhibits its transport to the nucleus (Orberg and Schaffer, 1987), presumably due to a significant change in the protein's conformation. Additionally, infected cell proteins were metabolically labelled with ^{35}S-methionine for immunoprecipitation studies of d21-infected cell proteins to determine if the deleted ICP8 was capable of complexing with UL37. CV-1 cells were infected with HSV, V37, and d21 at an MOI of 5, and infected cell proteins from nuclear and cytoplasmic extracts were separated by SDS-PAGE, blotted to nitrocellulose, and probed with anti-1.1 UL37 antiserum. Among the d21 infected cell proteins, UL37 protein was detected exclusively in the cytoplasm, indicating that in the absence of a functional ICP8 protein the UL37 protein is unable to be transported to the nucleus (Fig. 39). Again, UL37 was capable of being transported to the nucleus in HSV-infected cells, but not V37-infected cells. As a control, these nuclear and cytoplasmic extracts were analyzed for ICP8 by immunoblot (Fig. 39) that demonstrated the nuclear localization of ICP8 in HSV extracts, appearing as previously reported by Fenwick (1978), and the retention of the deleted ICP8 protein from d21 in the cytoplasmic fraction. To assess the purity of nuclear fractions, an

FIGURE 39

Immunoblot analysis of cytoplasmic and nuclear fractions from HSV, V37, and d21-infected cells. Cytoplasmic (C) and nuclear (N) fractions were prepared from HSV-1, V37, and d-21-infected cells and V37-infected cells harvested 24 HPI as previously described. Proteins were separated on a SDS-9% acrylamide gel and immunoblots probed with 1.1 and ICP8 (C-terminal peptide) polyclonal antisera (top and middle panels) and monoclonal anti-actin (bottom panel). Primary antibody binding was detected by alkaline phosphatase-conjugated anti-rabbit and anti-mouse antibodies, respectively, followed by addition of substrate. Molecular sizes of protein standards are indicated on the right.

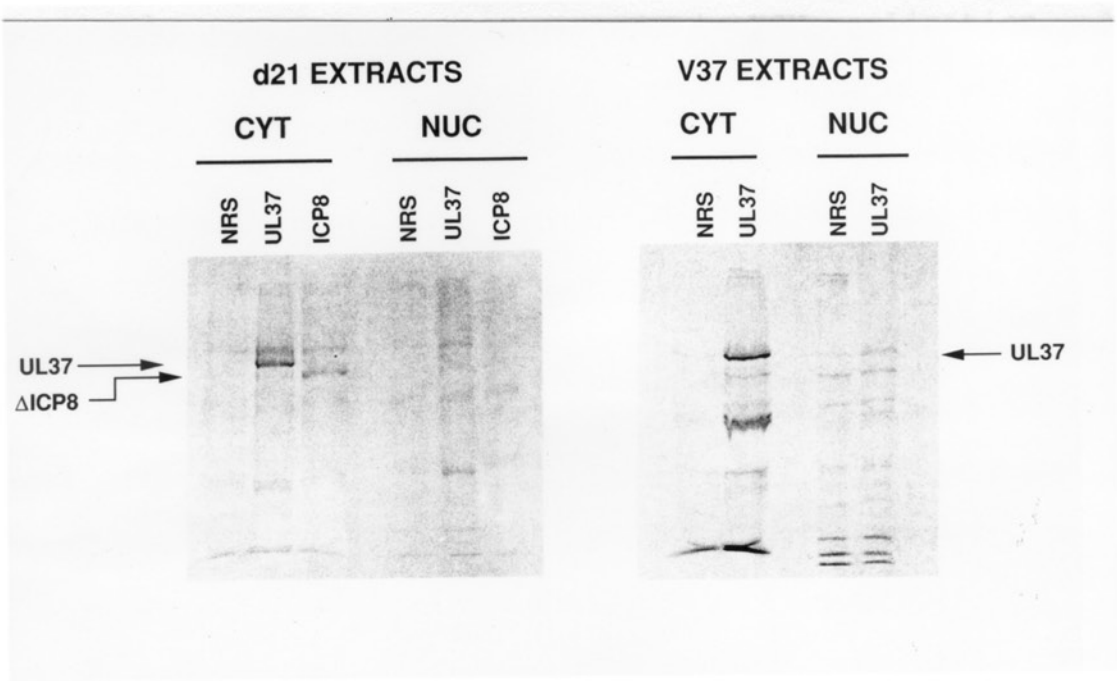

HSV V37 d21
N C N C N C
UL37
200
68
43
29
ICP8
ΔICP8
200
68
43
29
Actin
200
68
43
29

identical blot was probed for actin, a cytoskeletal protein located within the cytoplasm, using an anti-actin monoclonal antibody. Actin was detected predominantly in the cytoplasmic fractions (Fig. 39), indicating that the nuclear extracts were not grossly contaminated with this cytoplasmic protein. The purity of the nuclear preparations was also addressed in the immunoprecipitation studies described below.

Immunoprecipitation of UL37 and ICP8 proteins from d21 nuclear and cytoplasmic infected cell protein extracts using specific antisera for UL37 and ICP8 demonstrated precipitation of the individual proteins, 120 and 85 kD respectively, from the cytoplasm, but not the nucleus (Fig. 40), confirming the results observed by immunoblot analysis. As a control for the purity of the nuclear infected cell protein extracts, anti-1.1 UL37 antiserum was used to precipitate V37 protein which was found to be exclusively cytoplasmic. Co-precipitation of ICP8 and UL37 proteins from d21 cytoplasmic proteins was not observed, indicating that the deleted ICP8 protein was not capable of forming a stable complex with the UL37 protein. This finding was consistent with the results observed in immunoblot analysis of nuclear and cytoplasmic d21-infected cell proteins as well as SS DNA column chromatography using d21-infected cell proteins. The use of d21 in these studies demonstrates that the inhibition of complex formation between ICP8 and UL37

FIGURE 40

Immunoprecipitation reactions using cytoplasmic and nuclear fractions from d21 and V37-infected cells. Cytoplasmic (CYT) and nuclear (NUC) fractions were prepared from ^{35}S-methionine-labelled d21 and V37-infected cells 24 HPI. Immunoprecipitated proteins were separated on an SDS-9% polyacrylamide gel, fixed, and dried as described in Materials and Methods followed by image analysis on an Image Quant Phosphoimager. Immunoprecipitations using normal rabbit serum (NRS), 1.1 antiserum (UL37), and anti-ICP8 (C-terminal peptide) antiserum (ICP8) are shown.


d21 EXTRACTS
CYT
NUC
NRS
UL37
ICP8
NRS
UL37
ICP8
UL37
ΔICP8
V37 EXTRACTS
CYT
NUC
NRS
UL37
NRS
UL37
UL37

abrogates UL37's ability to bind DNA and localize to the nucleus which is observed in HSV-infected cells.

IV. Cell lines & UL37 recombinant viruses

A long range goal of our laboratory is to investigate the function(s) of the UL37 protein in HSV replication. As a first step towards this goal, generation of a viral recombinant deficient in UL37 expression would determine the essential or nonessential nature of the UL37 gene in the HSV lytic replication cycle in cell culture. Should the UL37 gene be essential for viral replication in a standard cell culture system, the use of a host-range (*hr*) mutant in the UL37 gene would be instrumental in determining the stage of viral replication affected. As a part of this study, we generated plasmid constructs which would be useful in generating several different UL37 viral recombinants, attempted to produce cell lines expressing the UL37 protein for potential *hr* mutants, and made numerous attempts to isolate UL37 viral recombinants.

At the start of this study, two plasmids were constructed containing the UL37 gene--one containing the UL37 gene under the control of its native promoter, pNH37, for generation of cell lines, and one excluding the promoter region except for 92 bp upstream of the UL37 ATG, pHC37, for use in creating mutagenized versions of UL37. The construction of the pHC37 plasmid has been described previously (Results, Section I). The plasmid pNH37 was

constructed by cloning a 4.6 kb *Hin*dIII-*Nhe*I DNA fragment from pRB210 into the *Hin*dIII-*Xba*I sites of Bluescript SK+. The *Nhe*I site is located 1220 bp upstream of the UL37 ATG. The *Hin*dIII is located 3 bp downstream of the UL37 stop codon. Two plasmids encoding G418 resistance, pRB3464 and pRB3448 (with and without the HSV ICP4 gene, respectively), were each co-transfected with the pNH37 plasmid into Vero cells to produce UL37-expressing cell lines. Forty-eight separate G418 resistant cell lines were isolated. Characterization of these cell lines for UL37 expression by immunoblotting indicated no expression at the protein level (data not shown). The lack of UL37 protein could be due to the inability of the UL37 native promoter to be constitutively expressed; however, if the UL37 DNA was stably maintained, there is a strong possibility that infection with HSV would induce its expression. Unfortunately, dot blot analysis of the high molecular weight DNA from each of these cell lines, using the *Hin*dIII-*Cla*I fragement as a probe for UL37 DNA sequences, was found to be negative, and most recently PCR analysis of several of these cell lines also was negative (data not shown). In order to address the possibility that the UL37 DNA was lost while the G418 resistance plasmid DNA was maintained, the plasmid pJF36 was constructed which contained the G418 resistance marker co-linked on the same plasmid as the UL37 gene. The plasmid pJF36 was constructed by cloning the

*Hin*dIII DNA fragment from pRB3448 into the *Hin*dIII site of pNH37. Transfection of CV-1 cells with the pJF36 plasmid resulted in the isolation of 4 G418 resistant cell lines, designated CV37s. Although UL37 DNA sequences were not detectable by dot blot analysis of the CV37 DNAs, the G418 resistant phenotype gave a strong indication that UL37 DNA sequences might also be present. Several different approaches to induce UL37 expression were investigated including infection with HSV-2(G), HSV-2(333), and pseudorabiesvirus; however, the UL37 protein of the inducing viruses was indistinguishable from the UL37 of HSV-1. Also, induction of UL37 expression by transfection of plasmids encoding ICP4 or a combination of ICP4 and αTIF into the CV37 cell lines was unsuccessful.

Most recently, the CV37 cells were found to be negative for UL37 DNA sequences by PCR analysis. Prior to this result, these cells were used for numerous attempts at isolating a UL37 viral recombinant by co-transfection of infectious HSV-1 DNA and plasmids containing UL37 mutations and the screenable marker β-galactosidase, and the β-galactosidase$^+$ ICP6 mutant hrR3 (Goldstein and Weller, 1988b) was used as a positive control. On the basis of these negative results, we might conclude that a UL37 viral recombinant requires UL37 complementation by the cell, indicating that it is essential for viral replication.

Most recently, the plasmid pJF68 was constructed by the

insertion of an *Eco*RI linker at the *Cla*I site in pHC37 for subsequent insertion by a coworker in this laboratory of an *Eco*RI DNA fragment containing the CMV immediate early promoter, a strong heterologous promoter. The expression of UL37 directed by this promoter has been detected transiently in CV-1 cells, and isolation of a stably transfected cell line in our laboratory is under way.

Plasmids containing mutations in the UL37 gene include a UL37/β-galactosidase protein fusion (pJF55), a UL37 gene insertionally inactivated by a cassette containing a strong HSV β gene promoter (ICP6) fused to the β-galactosidase gene (pJF66), and a UL37 deletion (pJF35)(Fig. 41). The UL37 deletion in pJF35 was constructed by a BstE2 deletion of 2.7 kb out of the 3.5 kb UL37 DNA contained in pHC37. The use of *lac*Z sequences for mutation of UL37 necessitated the construction of pJF49, consisting of the UL37 *Nhe*I-*Hin*dIII fragment inserted in a plasmid devoid of *lac*Z DNA (pBR329). The plasmid pJF49 was used to construct pJF55, expressing a UL37/β-galactosidase protein fusion containing the N-terminal 412 amino acids of UL37, by insertion of *lac*Z in frame with the UL37 coding sequence at the *Bcl*I site within the UL37 gene. A *Bam*HI fragment from pD6p, containing an ICP6::*lac*Z cassette (the HSV ICP6 promoter and 59 N-terminal amino acids of ICP6 fused to β-galactosidase), was also inserted into the *Bcl*I site of UL37 in pJF49. The advantage of the pJF66 construction is the expression of the

FIGURE 41

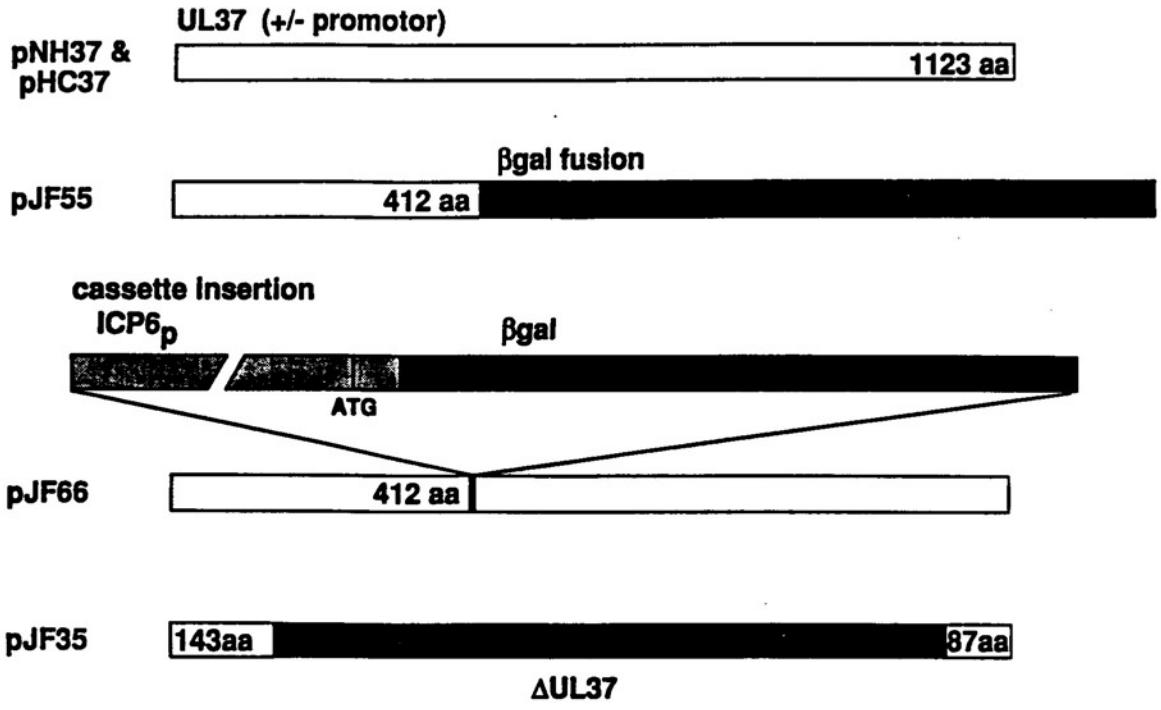
Schematic summary of mutations constructed in the UL37 gene to be used in production of viral recombinants.

pHC37 and pNH37 served as a source of the UL37 gene (including 92 bp and approximately 1 kb upstream sequences, respectively) for other plasmid constructions. The open box represents the full-length UL37 protein.

pJF55 contains the *Nhe*I-*Hin*dIII fragment from pNH37 with a *lac*Z insertion at the *Bcl*I site (in frame with UL37 coding sequence). pJF14 served as a source of *lac*Z as a BamHI fragment.

pJF66 contains the *Nhe*I-*Hin*dIII fragment from pNH37 with an ICP6::*lac*Z cassette inserted at the BclI site. The start codon of the ICP6-βgalactosidase fusion protein is indicated. ICP6 sequences upstream of the ATG are much greater than illustrated (1.2 kb, broken region).

pJF35 has a 2.7 kb deletion of a *Bst*E2 fragment from pHC37.


UL37 (+/- promotor)
pNH37 &
pHC37
1123 aa
βgal fusion
pJF55
412 aa
cassette insertion
ICP6p
βgal
ATG
pJF66
412 aa
pJF35
143aa
87aa
ΔUL37

screenable marker from an independent transcript; this cassette has been used successfully in the generation of an *hr* mutant in the HSV UL52 gene (Goldstein and Weller, 1988b). Both pJF55 and pJF66 demonstrate a β-galactosidase$^+$ phenotype in *E.coli*, indicating functional expression of β-galactosidase. Once a UL37-complementing cell line is produced, the use of the pJF55 and pJF66 plasmids will facilitate the efficient isolation of a UL37$^-$ β-galactosidase$^+$ viral recombinant (blue plaques with X-gal). The plasmid pJF35 can then be used to create a deletion, most likely a null mutation, in UL37 gene by co-transfection with the blue plaque viral DNA, screening for white plaques from a background of blue plaques.

DISCUSSION

In this report, by expression of the UL37 gene *in vitro* and the generation of UL37-specific antisera, we have shown that the predicted protein product of the previously uncharacterized HSV-1 UL37 gene is expressed in HSV-1 infected cells. The UL37 protein has an apparent molecular mass of 120 kD on SDS-PAGE and is a nonstructural protein. The vaccinia expressed UL37 protein, V37, is indistinguishable by immunoblot from the HSV-1 UL37 protein. UL37 is expressed in the absence of of viral DNA replication and reaches its maximum levels late in infection, classifying it as a $\gamma 1$ gene. The UL37 protein co-elutes from both SS and DS DNA columns with the HSV ICP8 protein. By comparative studies using the UL37 vaccinia recombinant, V37, and an ICP8 mutant, d21, whose ICP8 protein is incapable of binding DNA or nuclear localization, we have demonstrated that the DNA binding ability and the nuclear localization exhibited by the UL37 protein in HSV-infected cells are due to its association with the ICP8 protein.

The fact that the size of the UL37 protein, 120 kD, expressed by *in vitro* translation, a vaccinia recombinant, and HSV-1 are in agreement with the size based on the predicted amino acid sequence, 120.5, would indicate that the protein undergoes little or no modification which would affect its migration on SDS-PAGE. Recently, phosphorylation of the UL37 protein has been demonstrated in HSV-1-infected

cells, occurring as early as 6 to 12 HPI (Allen Albright, personal communication). Since the UL37 protein was not observed among the previously reported profile of HSV phosphoproteins (Wilcox *et al*, 1980), it would appear that the detection of the phosphorylated form of the UL37 protein required a UL37-specific antiserum and that the phosphorylated UL37 protein is not abundantly phoshorylated compared to the other phoshoproteins. Whether the state of phosphorylation affects the ability of the UL37 protein to associate with ICP8 and/or bind DNA is currently being investigated in our laboratory.

The UL37 protein gradually increases in abundance during HSV replication and requires viral DNA replication for maximal expression, classifying UL37 as a $\gamma 1$ gene. These results agree with those of Anderson and coworkers (1981) who found that a 3.8 kb mRNA, mapping to the coordinates more recently defined as the UL37 ORF (McGeogh *et al*, 1988b), was detected in less abundance early in infection and greater abundance late in infection. Recent pulse-chase studies indicate that the rate of UL37 protein synthesis is steady from 6-12 HPI and then drops from 12-15 HPI (Allen Albright, personal communication); since the relative amount of UL37 protein increases in HSV-infected cells, the accumulation of the UL37 protein late in infection (15-24 HPI) indicates that it is relatively stable once it is synthesized. The mechanisms involved in both

activation of initial expression of γ1 (also referred to as βγ or leaky-late) genes and acceleration of their expression following DNA replication are poorly understood. At least three out of five γ proteins (ICP4, ICP0, and ICP27) are required for full transactivation of late genes (Watson and Clements, 1980; DeLuca and Schaffer, 1985; Sacks *et al*, 1985; O'Hare and Hayward, 1985; Rice and Knipe, 1990; Cai and Schaffer, 1992); however, with the exception of ICP4 binding sites within the gD promoter (Faber and Wilcox, 1986; Beard *et al*, 1986; Michael *et al*, 1988; Tedder *et al*, 1989; Pizer *et al*, 1991), direct binding of α proteins to γ1 gene promoters has not been observed. Recently, Chen and coworkers (1992) have identified two copies of a "leaky-late binding site" (LBS), within the promoter sequence of the UL37 gene. The LBS was identified by footprinting studies using the promoter of ICP5, another HSV-1 γ1 gene, encoding the major capsid protein of HSV-1, and found to be important for transactivation of the ICP5 gene in transient transfection studies. They report that the LBS exists in other HSV-1 (gD, gB, and UL46), CMV, retrovirus, and cellular promoters and that a ubiquitous cellular transcriptional factor called YY1 or common factor-1 (also called NF-E1, δ, and UCRBP) binds the LBS. This is the first report of γ1-specific cis-element involved in regulation of γ1 gene expression of some HSV genes such as UL37.

Since the majority of known HSV-1 structural proteins

belong to either the γ1 or γ2 gene classes, classification of UL37 as a γ1 gene whose expression achieves maximum levels very late in infection would suggest that UL37 is a structural gene. However, analysis of intact, isolated HSV-1 virus particles indicates that within the limits of detection, the UL37 protein is not a component of the virion. While we cannot rule out the presence of minor amounts of UL37 protein in our preparations, the protein is clearly not a major component of the HSV-1 virion although it is present in readily detectable amounts in infected cells. The most likely possibility, therefore, is that the UL37 gene product is a nonstructural protein involved in late events of HSV replication.

Analysis of the predicted amino acid sequence of the UL37 protein reveals homology between the proteins encoded by the HSV UL37 gene and VZV gene 21, indicating that UL37 is well conserved among the neurotropic human herpesviruses. The genetic similarity throughout the entire length of these proteins may translate into a similarity in structure, and they may provide similar functions in viral replication. However, the presence of a potential ATP-binding site is unique to the UL37 protein sequence, and its significance has yet to be established. This motif has been reported for several known ATP-binding proteins such as the *E.coli* tyrosyl tRNA synthetase and porcine lactate dehydrogenase (Bradley, *et al*, 1987). The motif relies heavily on a

predicted secondary structure of (β strand)-GXXXXG-(α helix)-(β strand) which is contained in the UL37 amino acid sequence. The existence of a putative ATP-binding site suggests that the UL37 protein may be involved in an energy-driven step in late viral replication events.

In this report, we provide the first evidence of an interaction between the UL37 and ICP8 proteins of HSV-1. The direct analysis of such an association has been greatly hampered by the fact that these two proteins co-migrate on SDS-PAGE and are inseparable by numerous biochemical techniques. The use of specific antisera to identify each of these proteins has revealed the presence of the UL37 protein co-eluting with the ICP8 protein in fractions from SS and DS DNA columns which was undetectable by previous investigators (Bayliss *et al*, 1975; Powell and Purifoy, 1976; Purifoy and Powell, 1976; Knipe *et al*, 1982; Ruyechan and Weir, 1984). However, the purity of ICP8 protein preparations which have been used for biochemical studies of its properties is not entirely in question since ICP8 is very abundant early in infection at the time that HSV-infected cell proteins are generally harvested (4-6 HPI) for purification, and the distinct kinetics of UL37 expression indicates it is present at low levels at that time in the HSV lytic replication cycle. In fact, a polyclonal antiserum generated using purified ICP8 from SS DNA column chromatography which was used in this study shows no

reactivity against the UL37 protein expressed by V37 (data not shown) even though UL37 can be detected by immunoblot in similarly purified ICP8 preparations (data not shown). This may suggest that either the UL37 in these preparations is not very immunogenic or that the UL37 protein is present in very small quantities. Currently, without a means of distinguishing between these two proteins other than by the use of specific antisera which show differing affinities for their respective proteins, no conclusions can be made about the stoichiometry of the UL37-ICP8 complex in HSV-infected cells. The use of truncated versions of either protein such as the UL37 fusion proteins available in our laboratory may provide a means to differentiate between the proteins, should they be capable of complex formation, based on size. Attempts to reconstitute the complex *in vitro* by simply mixing purified ICP8 and V37 proteins have been unsuccessful, but this is typically the case with other protein complexes such as the HSV UL5/UL8/UL52 helicase/primase complex and DNAPol/UL42 complex (Mark Challberg, personal communication). Co-expression within a system such as *E.coli* or baculovirus, co-infection with vaccinia recombinants expressing each protein, or co-translation *in vitro* would provide a means for studying this complex formation. Preliminary *in vitro* translation studies within our laboratory suggest that the UL37-ICP8 complex forms as the UL37 protein is translated in the presence of pre-formed

ICP8 protein (Frank Jenkins, personal communication); these conditions would be expected during HSV replication based on kinetics of expression of these two proteins.

By comparative studies using HSV, V37, and d21-infected proteins, we have demonstrated that the DNA binding ability exhibited by the UL37 protein is absolutely dependent upon the presence of a functional ICP8 protein. Furthermore, the interaction between these two proteins facilitates the nuclear localization of the UL37 protein. ICP8 expressed in cells transfected with a plasmid encoding ICP8 localizes to the nucleus (Knipe and Smith, 1986). The carboxy-terminal 28 residues of ICP8 can function independently as a nuclear localization signal when covalently linked to pyruvate kinase expressed as a fusion protein (Gao and Knipe, 1991). Our results indicate that the noncovalent association between the ICP8 and UL37 proteins is sufficient for localization of the UL37 protein to the cell nucleus and that the ICP8 protein serves as a carrier molecule for UL37 nuclear localization. The development of a monoclonal antibody directed against UL37 for immunofluorescence studies is greatly desired to investigate whether the intranuclear destination of the UL37 protein continues to be directed by the ICP8 protein to the previously described replication compartments (de Bruyn Kops and Knipe, 1988) once the complex reaches the nucleus.

The functional significance of the UL37-ICP8

interaction in the HSV lytic replication cycle is not clear based on the multifunctional nature of the ICP8 protein. The requirement for ICP8 in HSV DNA replication early in infection has been firmly established by genetic studies using both ts and deletion mutants in ICP8 (Conley *et al*, 1981; Weller *et al*, 1983; Leinbach *et al*, 1984; Gao *et al*, 1988; Gao and Knipe, 1989). ICP8 is also required for replication of an *ori*-containing plasmid in a transient transfection system (Wu *et al*, 1988). Based on its biochemical properties, it is assumed that the function of the ICP8 protein is analogous to other helix-destabilizing proteins--that it binds DNA at single-stranded regions of the replication fork, stabilizing a multiprotein/DNA complex. It also appears that ICP8 is responsible for organizing the DNA replication complexes at discrete sites within the nucleus (de Bruyn Kops and Knipe, 1988). It is unlikely that UL37 plays a role in such early stages of viral DNA replication since maximal production of the UL37 protein does not occur until after the onset of viral DNA replication. However, the complexing of UL37 to the ICP8 protein may possibly result in a modification of ICP8 function late in viral replication. Since levels of ICP8 reach peak levels early during in infection, but remain high through late times, it is reasonable to think that ICP8 may play a different role late in infection. Other helix-destabilizing proteins, or single-strand binding proteins

(SSBs), have been reported to promote transcription of viral late genes including adenovirus DNA binding protein (E2A)(Chang and Schenk, 1990), T4 bacteriophage gene 32 protein (gp32)(Gauss *et al*, 1987; Herendeen *et al*, 1989; Herendeen *et al*, 1990), and *E.coli* SSB (Haynes and Rothman-Denes, 1985). Gao and Knipe (1991) recently describe a trans-dominant mutant form of the ICP8 protein (d105) that inhibits the expression of several late proteins during HSV infection (independent of the block in DNA synthesis), indicating that ICP8 is involved in stimulation of late gene expression. They propose that at late times after infection, the ICP8 protein binds to newly synthesized progeny DS DNA and holds it in a form which is optimal for transcription. This may occur by the binding of ICP8 to small single-stranded regions and keeping promoter regions open for transcription or by binding specific structures or sequences in late promoters. They suggest that ICP8 may require interactions with other viral and/or cellular proteins in order to recognize late gene promoters.

Potential mechanisms involved in stimulation of late gene transcription by HSV ICP8 and the adenovirus DNA binding protein, E2A, have been speculative at best and are based mainly on our current understanding of bacteriophage T4 DNA replication proteins and their effects on transcription. The studies on the T4 proteins provide evidence for the coupling of DNA replication with late gene

transcription. Two phage-encoded proteins, gp33 and gp55, compete with sigma factor-70 for binding on the *E.coli* RNA polymerase core, creating a T4-modified RNA polymerase which demonstrates a late promoter specificity (Herendeen *et al*, 1990). Three DNA polymerase accessory proteins, gp 44/62 and 45, form a complex which stimulates the opening of distal late promoters by serving as a "mobile enhancer" which either moves along with the DNA replication fork or binds at breaks in the non-transcribed DNA strand (Herendeen *et al*, 1989). The protein complex recognizes a DNA structure rather than a nucleotide sequence. The transcriptional enhancement by this complex requires an ATPase activity and can occur hundreds of base pairs away from its DNA binding site. The T4 helix-destabilizng protein (or single-strand binding protein, SSB), gp32, also can participate in activating transcription by making the gp44/62 and 45 protein complex bind more tightly. T4 gp32 increases the affinity of the complex for its binding site in vitro and can reverse the inhibitory effects of another T4-encoded protein, gp43, which competes with the complex for binding sites (Herendeen *et al*, 1989). In addition, some gp32 *ts* mutants show a greater defect in late gene expression than in DNA replication, similar to the d105 mutant of ICP8, suggesting that gp32 can independently effect transcriptional activation, apart from effects on DNA replication (Gauss *et al*, 1987). The gp32 protein,

therefore, is one among several T4 replication proteins which are involved in the transcriptional activation of late promoters.

Gauss *et al* (1987) have proposed that the T4 gp32 protein can bind preferentially to specific nucleic acid ligands created by secondary structures. This proposal is based on the ability of T4 gp32 to autoregulate its own translation by binding an RNA pseudoknot/A-U rich region in its own mRNA (Russel *et al*, 1976; Gold *et al*, 1976; Shamoo *et al*, 1991). The structure of the unwound DNA in an open promoter complex is unknown, and some regions of DNA might mimic a "pseudoknot-like" structure. The translational regulation of gp32 only occurs once the protein has bound SS DNA and is present in excess at late times during infection (Gold *et al*, 1976). Whether it is possible for other helix-destabilizing proteins such as HSV ICP8 to bind secondary structures in DNA and/or RNA either independently or in conjunction with other viral proteins, such as the case with T4 gp32, is an intriguing possibility. Such interactions may include other viral proteins such as UL37 and/or cellular proteins.

ICP8 has been reported to interact with HSV proteins other than UL37 such as the alkaline exonuclease protein and the DNAPol (Littler *et al*, 1983; Vaughn *et al*, 1984; Thomas *et al*, 1992). These three proteins have been detected among proteins bound to an ICP8 immunoaffinity column (Vaughn *et*

al, 1984). The ICP8 interaction with DNAPol most likely occurs within the DNA replication complexes since both proteins localize to replication compartments (Knipe, 1989) and ts mutants in ICP8 affect DNAPol activity (Littler *et al*, 1983) and alter its sensitivity to inhibitory drugs (Chiou *et al*, 1985). The fact that two well characterized DNA replication proteins interact is not surprising. However, the role of the ICP8-alkaline exonuclease interaction is less clear. While ICP8 localizes exclusively to nuclear replication compartments, alkaline exonuclease localizes to the nucleus independent of the ICP8 protein and can be found in both the replication compartments and in other sites within the nucleus (Thomas *et al*, 1992). Weller and coworkers (1990) report that a deletion mutant in alkaline exonuclease produces wild-type levels of viral DNA and late viral proteins, but fails to process viral DNA into capsids (reviewed in Weller, 1991). Chou and Roizman (1989) have purified two distinct protein complexes (V2 and V4) which bind viral DNA at separate signals (pac-1 and pac-2) required for cleavage of viral DNA. A single protein in the V2 complex was purified and identified as the alkaline exonuclease (or DNase) by the use of a monoclonal antibody; however, the alkaline exonuclease appeared to bind DNA in a sequence-independent manner and, therefore, they suggested that a sequence-specific activity may be directed by interaction with another protein(s) which was not present

within the purified complex. Since alkaline exonuclease is detectable as early as 2 HPI and ts mutants in ICP8 affect alkaline exonuclease activity (Littler *et al*, 1983), it has been suggested that alkaline exonuclease may be involved in both early events in DNA replication and late events in DNA processing into capsids. Whether ICP8 plays a role in such late events has yet to be determined. Such a role is difficult to assess using ICP8 mutants since ICP8 is essential for viral DNA replication.

Clearly, the ICP8 protein is an essential, multifunctional protein in the HSV replication cycle. The UL37 protein complexes with ICP8 late in infection and potentially may be involved in any of the postulated "late" ICP8 functions with UL37 serving as an accessory protein which alters or assists ICP8 function. The functional significance of this complex is now a focus of study within our laboratory. The UL37-ICP8 complex may be involved in late stages of DNA replication, late gene regulation, or processing of progeny viral DNA. As a regulatory protein complex, the general transcriptional machinery may be affected or the DNA binding specificity of ICP8 may be altered allowing it to transactivate specific viral genes.

The use of HSV-1 viral recombinants with separate mutations in each of these proteins is critical to understanding the significance of this complex in HSV viral replication. As a part of this project, several UL37

constructs were generated for use in producing viral recombinants deficient in UL37 expression. Numerous attempts to isolate a UL37 recombinant were unsuccessful in cells which did were incapable of complementing such mutations, suggesting that UL37 may be essential for viral replication in cell culture. Since the production of a complementing cell line was repeatedly unsuccessful, this raised the possibility that UL37 expression is toxic to the cells. Further attempts at creating a UL37-complementing cell line are in progress in our laboratory. Alternatively, generation of conditional lethal mutants, which require more labor-intensive screening, may be a valuable approach. Ultimately, the generation of UL37 recombinants will be useful for determining which stage of the HSV lytic replication the UL37 protein and/or the UL37-ICP8 complex plays a role.

REFERENCES

Anderson, K.P, R.J. Frink, G.B. Devi, B.H. Gaylord, R.H. Costa, and E.K. Wagner. 1981. Detailed characterization of the mRNA mapping in the *Hin*dIII fragment K region of the herpes simplex virus type 1 genome. J. Virol. **37**:1011-1027.

Andrewes, C.H., and E.A. Carmichael. 1930. A note on the presence of antibodies to herpesvirus in post-encephalic and other human sera. Lancet **1**:857-858.

Baer, R., A.T. Bankier, M.D. Biggin, P.L. Deininger, P.J. Farrell, T.J. Gibson, G. Hatfull, G.S. Hudson, S.C. Satchwell, C. Seguin, P.S. Tuffnell, and B.G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207-211.

Baringer, J.R. 1974. Recovery of herpes simplex virus from human sacral ganglions. N. Engl. J. Med. **291**:828-830.

Baringer, J.R. 1975. Herpes simplex virus infection of nervous tissue in animals and man. Progr. Med. Virol. **20**:1-26.

Baringer, J.R. 1976. The biology of herpes simplex virus infection in humans. Surv. Ophthalmol. **21(2)**:171-174.

Baringer, J.R., and P. Swoveland. 1973. Recovery of herpes-simplex virus from human trigeminal ganglions. N. Engl. J. Med. **288**:648-650.

Batterson, W., D. Furlong, and B. Roizman. 1983. Molecular genetics of herpes simplex virus. VIII. Further characterization of a temperature-sensitive mutant defective in release of viral DNA and in other stages of the viral reproductive cycle. J. Virol. **45**:397-407.

Bayliss, G.J., H.S. Marsden, and J. Hay. 1975. Herpes simplex virus proteins: DNA-binding proteins in infected cells and in the virus structure. Virology. **68**:124-134.

Beard, P., S. Faber, K.W. Wilcox, and L.I. Pizer. 1986. Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA **83**:4016-4020.

Benjamin, T.L. 1970. Host range mutants of polyoma virus. Proc. Natl. Acad. Sci. USA **67**:394-399.

Ben-Porat, T., A.S. Kaplan, B. Stehn, and A.S. Rubenstein. 1976. Concatameric forms of intracellular herpesvirus DNA. Virology. **69**:547-560.

Bradley, M.K., T.F. Smith, R.H. Lathrop, D.M. Livingston, and T.A. Webster. 1987. Consensus topography in the ATP binding site of the simian virus 40 and polyomavirus large tumor antigens. Proc. Natl. Acad. Sci. USA **84**:4026-4030.

Buchman, T.G., B. Roizman, and A.J. Nahmias. 1979. Demonstration of exogenous genital reinfection with herpes simplex virus type 2 by restriction endonuclease fingerprinting of viral DNA. J. Infec. Dis. **140**:295-304.

Burnet, F.M., and S.W. Williams. 1939. Herpes simplex: new point of view. Med. J. Aust. **1**:637-642.

Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.

Cai, W., and P.A. Schaffer. 1991. A cellular function can enhance gene expression and plating efficiency of a mutant defective in the gene for ICP0, a transactivating protein of herpes simplex virus type 1. J. Virol. **65**:4078-4090.

Cai, W., and P.A. Schaffer. 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J. Virol.

66:2904-2915.

Campbell, M.E.M., J.W. Palfreyman, and C.M. Preston. 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate early transcription. J. Mol. Biol. **180**:1-19.

Carton, C.A., and E.D. Kilbourne. 1952. Activation of latent herpes simplex by trigeminal sensory-root section. N. Engl. J. Med. **246**:172-176.

Chakrabarti, S., K. Brechling, and B. Moss. 1985. Vaccinia virus expression vectors: coexpression of β-galactosidase provides visual screening of recombinant virus plaques. Mol. Cell. Biol. **5**:3403-3409.

Challberg, M.D. 1986. A method for identifying the viral genes required for herpes virus DNA replication. Proc. Natl. Acac. Sci. USA **83**:9094-9098.

Challberg, M.D., and T.J. Kelly. 1989. Animal virus DNA replication. Ann. Rev. Biochem. **58**:671-717.

Chang, L. and T. Schenk. 1990. The adenovirus DNA-binding protein stimulates the rate of transcription directed by

adenovirus and adeno-associated virus promoters. J. Virol. **64**:2103-2109.

Chase, J.W., and K.R. Williams. 1986. Single-stranded DNA binding proteins required for DNA replication. Ann. Rev. Biochem. **55**:103-136.

Chee, M.S., A.T. Bankier, S. Beck, R. Bohni, C.M. Brown, R. Cerny, T. Horsnell, C.A. Hutchison III, T. Kouzarides, J.A. Martignetti, E. Preddie, S.C. Satchwell, P. Tomlinson, K.M. Weston, and B.G. Barrell. 1990. Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. **154**:125-170.

Chen, S., L. Mills, P. Perry, S. Riddle, R. Wobig, R. Lown, and R.L. Millette. 1992. Transactivation of the major capsid protein gene of herpes simplex virus type 1 requires a cellular transcription factor. J. Virol. **66**:4304-4314.

Chiou, H.C., S.K. Weller, and D.M. Coen. 1985. Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. Virology. **145**:213-226.

Chou, J., and B. Roizman. 1989. Characterization of DNA sequence-common and sequence-specific proteins binding to

cis-acting sites for cleavage of the terminal *a* sequence of the herpes simplex virus 1 genome. J. Virol. **63**:1059-1068.

Conley, A.J., D.M. Knipe, P.C. Jones, and B. Roizman. 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of γ polypeptides. J. Virol. **37**:191-206.

Cook, M.L., and J.G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infec. Immun. **7**:272-288.

Corey, L., and P.G. Spear. 1986. Infections with herpes simplex viruses. N. Engl. J. Med. **314**:686-691.

Dargan, D.J. 1986. The structure and assembly of herpesviruses, p. 359-437. *In* Electron microscopy of proteins. 5. Viral structure. Academic Press Inc., London.

Darlington, R.W., and L.H. Moss III. 1968. Herpesvirus envelopment. J. Virol. **2**:48-55.

Davison, A.J., and D.J. McGeogh. 1986. Evolutionary comparisons of the S segments in the genomes of herpes

simplex virus type 1 and varicella-zoster virus. J. Gen. Virol. **67**:597-611.

Davison, A.J., and J.E. Scott. 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. **67**:1759-1816.

Davison, A.J., and P. Taylor. 1987. Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. **68**:1067-1079.

de Bruyn Kops, A., and D.M. Knipe. 1988. Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell. **55**:857-868.

Deiss, L.P., J. Chou, and N. Frenkel. 1986. Functional domains within the *a* sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. **59**:605-618.

DeLuca, N.A., and P.A. Shaffer. 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. Cell. Biol. **5**:1997-2008.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A

comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387-395.

Dingwall, C., and R.A. Laskey. 1986. Protein import into the cell nucleus. Ann. Rev. Cell Biol. **2**:367-390.

Dodd, K., L.M. Johnston, and G.J. Buddingh. 1938. Herpetic stomatitis. J. Pediatric. **12**:95-102.

Ejercito, P.M., E.D. Kieff, and B. Roizman. 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2**:357-364.

Faber, S.W., and K.W. Wilcox. 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nuc. Acids Res. **14**:6067-6083.

Field, H.J., S.E. Bell, G.B. Elion, A.A. Nash, and P. Wildy. 1979. Effect of acycloguanosine treatment on acute and latent herpes simplex infections in mice. Antimicrob. Agents Chemother. **15**:554-561.

Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6-13.

Fenwick, M.L., M.J. Walker, J.M.Petkevich. 1978. On the association of virus proteins with the nuclei of cells infected with herpes simplex virus. J. Gen. Virol. **39**:519-529.

Frink, R.J., R. Eisenberg, G. Cohen, and E.K. Wagner. 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. **45**:634-647.

Fuller, A.O., and W. Lee. 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. J. Virol. **66**:5002-5012.

Gallardo, E. 1943. Primary herpes simplex keratitis. Arch. Ophthalmol. **30**:217-220.

Gallo, M.L., D.H. Jackwood, M. Murphy, H.S. Marsden, and D.S. Parris. 1988. Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of its association with the virus-encoded DNA polymerase. J. Virol. **62**:2874-2883.

Gao, M., J. Bouchey, K. Curtin, and D.M. Knipe. 1988. Genetic identification of a portion of the herpes simplex

virus ICP8 protein required for DNA binding. **163**:319-329.

Gao, M., and D.M. Knipe. 1989. Genetic evidence for multiple nuclear functions of the herpes simplex virus ICP8 DNA-binding protein. J. Virol. **63**:5258-5267.

Gao, M., and D.M. Knipe. 1991. Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. J. Virol. **65**:2666-2675.

Gauss, P., K.B. Krassa, D.S. McPheeters, M.A. Nelson, and L. Gold. 1987. Zinc(II) and the single-stranded DNA binding protein of bacteriophage T4. Proc. Natl. Sci. USA **84**:8515-8519.

Gibson, W., and B. Roizman. 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. J. Virol. **10**:1044-1052.

Godowski, P.J., and D.M. Knipe. 1983. Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. **47**:478-486.

Godowski, P.J., and D.M. Knipe. 1985. Identification of a

herpes simplex virus function that represses late gene expression from parental viral genomes. J. Virol. **55**:357-365.

Godowski, P.J., and D.M. Knipe. 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA. **83**:256-260.

Gold, L., P.Z. O'Farrell, and M. Russel. 1976. Regulation of gene 32 expression during bacteriophage T4 infection of *Escherichia coli*. J. Biol. Chem. **251**:7251-7262.

Goldstein, D.J., and S.K. Weller. 1988a. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lac*Z insertion mutant. J. Virol. **62**:196-205.

Goldstein, D.J., and S.K. Weller. 1988b. An ICP6::lacZ insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. **62**:2970-2977.

Goodrich, L.D., F.J. Rixon, and D.S. Parris. 1989. Kinetics of expression of the gene encoding the 65-

kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. **63**:137-147.

Graham, F.L., and A.J. Van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology. **52**:456-467.

Greenberg, M.S., V.J. Brightman, and I.I. Ship. 1969. Clinical and laboratory differentiation of recurrent intraoral herpes simplex virus infections following fever. J. Dent. Res. **48**:385-391.

Greenberg, M.S., H. Friedman, S.G. Cohen, S.H. Oh, L. Laster, and S. Starr. 1987. A comparative study of herpes simplex infections in renal transplant and leukemic patients. J. Infec. Dis. **156**:280-287.

Gupte, S.S., J.W. Olson, and W.T. Ruyechan. 1991. The major herpes simplex virus type-1 DNA-binding protein is a zinc metalloprotein. J. Biol. Chem. **266**:11413-11416.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557-580.

Harlow, E., and D. Lane. 1988. "Antibodies: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor,

N.Y.

Hay, J., C.R. Roberts, W.T. Ruyechan, and A.C. Stevens. 1987. Herpesviridae, p. 391-405. *In* M.V. Nermut and A.C. Steven (ed), Animal Virus Structure. Elsevier Science Publishers.

Haynes, L.L., and L.B. Rothman-Denes. 1985. N4 virion RNA polymerase sites of transcription initiation. Cell. **41**:597-605.

Heine, J.W., P.G. Spear, and B. Roizman. 1972. Proteins specified by herpes simplex virus. VI. Viral proteins in the plasma membrane. J. Virol. **9**:431-439.

Herendeen, D.R., G.A. Kassavetis, J. Barry, B.M. Alberts, and E.P. Geiduschek. 1989. Enhancement of bacteriophage T4 late transcription by components of the T4 replication apparatus. Science. **245**:952-958.

Herendeen, D.R., K.P. Williams, G.A. Kassavetis, and E.P. Geiduschek. 1990. An RNA polymerase-binding protein that is required for communication between an enhancer and a promoter. Science. **248**:573-578.

Herold, B.C., D. WuDunn, N. Soltys, and P.G. Spear. 1991.

Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. **65**:1090-1098.

Hill, T.J. 1985. Herpes simplex virus latency, p. 175-240. *In* B. Roizman (ed), The herpsviruses, vol. 3. Plenum Press, New York.

Honess, R.W., and B. Roizman. 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in the infected cell. J. Virol. **12**:1347-1365.

Honess, R.W., and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. **14**:8-19.

Honess, R.W., and B. Roizman. 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA **72**:1276-1280.

Jacob, R.J., L.S. Morse, and B. Roizman. 1979. Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail

concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements in viral DNA. J. Virol. **29**:448-457.

Javier, R.T., J.G. Stevens, V.B. Dissette, and E.K. Wagner. 1988. A herpes simplex virus transcript abundant in latently infected neurons is dispensable for establishment of the latent state. Virology. **166**:254-257.

Jenkins, F.J., and B. Roizman. 1986. Herpes simplex virus 1 recombinants with noninverting genomes frozen in different isomeric arrangements are capable of independent replication. J. Virol. **59**:494-499.

Johnson, D.C., and P.G. Spear. 1982. Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. **43**:1102-1112.

Johnson, D.C., and P.G. Spear. 1983. O-linked oligosaccharides are acquired by herpes simplex virus glycoproteins in the Golgi apparatus. Cell. **32**:987-997.

Kalderon, D., B.L. Roberts, W.D. Richardson, and A.E. Smith. 1984. A short amino acid sequence able to specify nuclear location. Cell. **39**:499-509.

Kieff, E.D., S.L. Bachenheimer, and B. Roizman. 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J. Virol. **8**:125-129.

Klein, R.J. 1976. Pathogenetic mechanisms of recurrent herpes simplex viral infections. Arch. Virol. **51**:1.

Knipe, D.M. 1989. The role of viral and cellular nuclear proteins in herpes simplex virus replication. Adv. Vir. Res. **37**:85-123.

Knipe, D.M., M.P. Quinlan, and A.E. Spang. 1982. Characterization of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1. J. Virol. **44**:736-741.

Knipe, D.M., and J.L. Smith. 1986. A mutant herpesvirus protein leads to a block in nuclear localization of other viral proteins. Mol. Cell. Biol. **6**:2371-2381.

Knipe, D.M., and A.E. Spang. 1982. Definition of a series of stages in the association of two herpesviral proteins with the cell nucleus. J. Virol. **43**:314-324.

Knotts, F.B., M.L. Cook, and J.G. Stevens. 1974.

Pathogenesis of herpetic encephalitis in mice after ophthalmic inoculation. J. Infec. Dis. **130**:16-27.

Kornberg and Baker. 1992. DNA-binding proteins, p. 323-354. *In* DNA Replication, 2nd ed. W.H. Freeman and Co., New York.

Kristensson, K., E. Lycke, and J. Sjostrand. 1971. Spread of herpes simplex virus in peripheral nerves. Acta neuropath. **17**:44-53.

Kristensson, K., E. Lycke, M. Roytta, B. Svennerholm, and A. Vahlne. 1986. Neuritic transport of herpes simplex virus in rat sensory neurons *in vitro*. Effects of substances interacting with microtubular function and axonal flow [nocodazole, taxol, and erythro-9-3-(2-hydroxynonyl) adenine]. J. Gen. Virol. **67**:2023-2028.

Kristie, T.M., J.H. LeBowitz, and P.A. Sharp. 1989. The octamer-binding proteins form multi-protein - DNA complexes with the HSV αTIF regulatory protein. EMBO J. **8**:4229-4238.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.

Lee, C.K., and D.M. Knipe. 1983. Thermolabile in vivo DNA-binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. J. Virol. **46**:909-919.

Leetsma, J.E., M.B. Bornstein, R.D. Sheppard, and L.A. Feldman. 1969. Ultrastructural aspects of herpes simplex virus infection in organized cultures of mammalian nervous tissue. Lab. Invest. **20**:70-78.

Leib, D.A., D.M. Coen, C.L. Bogard, K.A. Hicks, D.R. Yager, D.M. Knipe, K.L. Tyler, and P.A. Schaffer. 1989a. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. **63**:759-768.

Leib, D.A., C.L. Bogard, M. Kosz-Vnenchak, K.A. Hicks, D.M. Coen, D.M. Knipe, and P.A. Schaffer. 1989b. A deletion mutant of the latency-associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. **63**:2893-2900.

Leinbach, S.S., J.F. Casto, and T.K. Pickett. 1984. Deoxyribonucleoprotein complexes and DNA synthesis of herpes simplex virus type 1. Virology. **137**:287-296.

Leinbach, S.S., and L.S. Heath. 1988. A carboxy-terminal peptide of the DNA-binding protein ICP8 of herpes simplex virus contains a single-stranded DNA-binding site. Virology. **166**:10-16.

Leinbach, S.S., and L.S. Heath. 1989. Characterization of the single-stranded DNA-binding domain of the herpes simplex virus protein ICP8. Biochim. Biophys. Acta. **1008**:281-286.

Ligas, M.W., and D.C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486-1494.

Littler, E., D. Purifoy, A. Minson, and K.L. Powell. 1983. Herpes simplex virus non-structural proteins. III. Function of the major DNA-binding protein. J. Gen. Virol. **64**:983-995.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. "Molecular cloning: a laboratory manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Marsden, H.S., M.E.M. Campbell, L. Haarr, M.C. Frame, D.S. Parris, M. Murphy, R.G. Hope, M.T. Muller, and C.M. Preston. 1987. The 65,000-M_r DNA-binding and virion *trans*-inducing

proteins of herpes simplex virus type 1. J. Virol **61**:2428-2437.

Matthews, R.E.F. 1982. Classification and nomenclature of viruses. Intervirology. **17**:1-200.

McGeogh, D.J. 1989. The genomes of the human herpes-viruses: contents, relationships, and evolution. Ann. Rev. Microbiol. **43**:235-265.

McGeogh, D.J., M.A. Dalrymple, A. Dolan, D. McNab, L.J. Perry, P. Taylor, and M.D. Challberg. 1988a. Structures of herpes simplex virus type 1 required for replication of virus DNA. J. Virol. **62**:444-453.

McGeogh, D.J., M.A. Dalrymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNab, L.J. Perry, J.E. Scott, and P. Taylor. 1988b. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69**:1531-1574.

McLennan, J.L., and G. Darby. 1980. Herpes simplex virus latency: the cellular location of virus in dorsal root ganglia and the fate of the infected cell following virus activation. J. Gen. Virol. **51**:233-243.

Mellerick, D.M., and N.W. Fraser. 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology. **158**:265-275.

Michael, N., D. Spector, P. Mavromara-Nazos, T.M. Kristie, and B. Roizman. 1988. The DNA-binding properties of the major regulatory protein α4 of herpes simplex viruses. Science. **239**:1531-1534.

Mocarski, E.S., and B. Roizman. 1982. Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. Cell. **31**:89-97.

Nahmias, A.J., and W.R. Dowdle. 1968. Antigenic and biologic differences in herpesvirus hominis. Progr. Med. Virol. **10**:110-159.

Nahmias, A.J., and B. Roizman. 1973. Infection with herpes-simplex viruses 1 and 2. N. Engl. J. Med. **289**:667-674, 719-725, 781-789.

Oakley, B.R., D.R. Kirsch, and N.R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105**:361-

363.

O'Hare, P., and G.S. Hayward. 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the *trans*-activation of delayed-early promoters. J. Virol. **53**:751-760.

Olivo, P.D., and M.D. Challberg. 1988. Herpes simplex virus DNA replication: identification of the essential genes and their products, p. 43-51. *In* T. Kelly and B. Stillman (ed.), Cancer cells, vol. 6. Eukaryotic DNA replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Olivo, P.D., and M.D. Challberg. 1991. Functional analysis of the herpes simplex virus gene products involved in DNA replication, p. 137-150. *In* E.K. Wagner (ed), Herpesvirus transcription and its regulation. CRC Press, Boca Raton.

Orberg, P.K., and P.A. Schaffer. 1987. Expression of herpes simplex virus type 1 major DNA-binding protein, ICP8, in transformed cell lines: complementation of deletion mutants and inhibition of wild-type virus. J. Virol. **61**:1136-1146.

Papavassiliou, A.G., K.W. Wilcox, and S.J. Silverstein. 1991. The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. EMBO J. **10**:397-406.

Para, M.F., R.B. Baucke, and P.G. Spear. 1980. Immunoglobulin G(Fc)-binding receptors on virions of herpes simplex virus type 1 and transfer of these receptors to the cell surface by infection. J. Virol. **34**:512-520.

Park, N.H., and D. Pavan-Langston. 1982. Effect of acyclovir, bromovinyldeoxyuridine, vidarabine, and L-lysine on latent ganglionic herpes simplex virus *in vitro*. Am. J. Med. **73(1A)**:151-154.

Peterslund, N.A. 1991. Herpesvirus infection: an overview of the clinical manifestations. Scand. J. Infect. **Suppl. 78**:15-20.

Pizer, L.I., R.D. Everett, D.G. Tedder, M. Elliott, and B. Litman. 1991. Nucleotides within both proximal and distal parts of the consensus sequence are important for specific DNA recognition by the herpes simplex virus regulatory protein ICP4. Nucleic Acids Res. **19**:477-483.

Poffenberger, K.L., and B. Roizman. 1985. A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. **53**:587-595.

Post, L.E., A.J. Conley, E.S. Mocarski, and B. Roizman. 1980. Cloning of reiterated and nonreiterated herpes simplex virus 1 sequences as BamHI fragments. Proc. Natl. Acad. Sci. USA **77**:4201-4205.

Post, L.E., S. Mackem, and B. Roizman. 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. Cell. **24**:555-565.

Powell, K.L., E. Littler, and D.J.M. Purifoy. 1981. Nonstructural proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. **39**:894-902.

Powell, K.L., and D.J.M. Purifoy. 1976. DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology. **7**:225-239.

Powell, K.L., and D.J.M. Purifoy. 1977. Nonstructural proteins in herpes simplex virus type 1. I. Purification of

the induced DNA polymerase. J. Virol. **24**:618-626.

Powell, K.L., and D.H. Watson. 1975. Some structural antigens of herpes simplex virus type 1. J. Gen. Virol. **29**:167-178.

Preston, C.M., M.C. Frame, and M.E.M. Campbell. 1988. A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell. **52**:425-434.

Purifoy, D.J.M., and K.L. Powell. 1976. DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. J. Virol. **19**:717-731.

Quinlan, M.P., L.B. Chen, and D.M. Knipe. 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell. **36**:857-868.

Rice, S.A., and D.M. Knipe. 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus α protein ICP27. J. Virol. **64**:1704-1715.

Roberts, B. 1989. Nuclear location signal-mediated protein transport. Biochim. Biophys. Acta. **1008**:263-280.

Robbins, J., S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1991. Two interdependent basic domains in nucleoplasmin nuclear targeting sequence: identification of a class of bipartite nuclear targeting sequence. Cell. **64**:615-623.

Rock, D.L., and N.W. Fraser. 1983. Detection of HSV-1 genome in central nervous system of latently infected mice. Nature (London). **302**:523-525.

Rock, D.L., and N.W. Fraser. 1985. Latent herpes simplex virus type 1 DNA contains 2 copies of the virion DNA joint region. J. Virol. **55**:849-852.

Rock, D.L., A.B. Nesburn, H. Ghiasi, J. Ong, T.L. Lewis, J.R. Lokensgard, and S.L. Wechsler. 1987. Detection of latency-related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. J. Virol. **61**:3820-3826.

Roizman, B. 1979. The structure and isomerization of herpes simplex virus genomes. Cell. **16**:481-494.

Roizman, B., L.E. Carmichael, F. Deinhardt, G. de-The, A.J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf. 1981. Herpesviridae: definition, provisional nomenclature, and taxonomy. **16**:201-217.

Roizman, B., and D. Furlong. 1974. The replication of herpesviruses, p.229-403. *In* H. Fraenkel-Conrat and R.R. Wagner (ed), Comprehensive Virology, vol.3. Plenum Press, New York.

Roizman, B., and A.E. Sears. 1990. Herpes simplex viruses and their replication, p. 1795-1841. *In* B.N. Fields, D.M. Knipe, *et al* (ed), Virology, 2nd ed. Raven Press, New York.

Russel, M., L. Gold, H. Morrissett, and P.Z. O'Farrell. 1976. Translational, autogenous regulation of gene 32 expression during bacteriophage T4 infection. J. Biol. Chem. **251**:7263-7270.

Ruyechan, W.T. 1983. The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. J. Virol. **46**:661-666.

Ruyechan, W.T., A. Chytil, and C.M. Fisher. 1986. In vitro characterization of a thermolabile herpes simplex virus DNA-binding protein. J. Virol. **59**:31-36.

Ruyechan, W.T., and A.C. Weir. 1984. Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. **52**:727-733.

Sacks, W.R., C.C. Greene, D.P. Aschman, and P.A. Schaffer. 1985. Herpes simplex virus type 1 ICP27 gene is an essential regulatory protein. J. Virol. **55**:796-805.

Sarmiento, M., M. Haffey, and P.G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B_2) in virion infectivity. J. Virol. **29**:1149-1158.

Scott, T.F.M., and A.J. Steigman. 1941. Acute infectious gingivostomatitis: etiology, epidemiology, and clinical picture of a common disorder caused by the virus of herpes simplex. J.A.M.A. **117**:999-1005.

Segal, A.L., A.H. Katcher, V.J. Brightman, and M.F. Miller. 1974. Recurrent herpes labialis, recurrent aphthous ulcers, and the menstrual cycle. **53**:797-803.

Shamoo, Y., K.R. Webster, K.R., Williams, and W.H. Konigsberg. 1991. A retrovirus-like zinc domain is essential for translational repression of bacteriophage T4 gene 32. J. Biol. Chem. **266**:7967-7970.

Sheldrick, P., and Berthelot, N. 1975. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symp. Quant. Biol. **39**:667-678.

Shieh, M.-T., D. WuDunn, R.I. Montgomery, J.D. Esko, and P.G. Spear. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. **116**:1273-1281.

Southern, K.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503-517.

Spear, P.G. 1985. Glycoproteins specified by herpes simplex viruses, p. 315-356. *In* B. Roizman (ed), The herpesviruses, vol. 3. Plenum Press, New York.

Spear, P.G., and B. Roizman. 1968. The proteins specified by herpes simplex virus. I. Time of synthesis, transfer into nuclei, and properties of proteins made in productively infected cells. Virology. **36**:545-555.

Spear, P.G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. **9**:431-439.

Spear, P.G., M. Wittels, A.O. Fuller, D. WuDunn, and R. Johnson. 1989. Herpes simplex virus: pathway of entry into cells, p. 163-175. *In* R.W. Compans, A. Helenius, and M.B.A. Oldstone (ed), Cell Biology of Virus Entry,

Replication, and Pathogenesis. Alan R. Liss, Inc., New York.

Spivack, J.G., and N.W. Fraser. 1987. Detection of herpes simplex virus type 1 transcripts during latent infection in mice. J. Virol. **61**:3841-3847.

Stackpole, C.W. 1969. Herpes-type virus of the frog renal adenocarcinoma. J. Virol. **4**:75-93.

Stanberry, L.R. 1986. Herpesvirus latency and recurrence. Prog. Med. Virol. **33**:61-77.

Steiner, I., J.G. Spivack, R.P. Lirette, S.M. Brown, A.R. MacLean, J.H. Subak-Sharpe, and N.W. Fraser. 1989. Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. **8**:505-511.

Steiner, I., J.G. Spivack, S.L. Deshmane, C.I. Ace, C.M. Preston, and N.W. Fraser. 1990. A herpes simplex virus type 1 mutant containing nontransinducing Vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. J. Virol. **64**:1630-1638.

Stern, S., M. Tanaka, and W. Herr. 1989. The Oct-1 homeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. Nature. **341**:624-630.

Stevens, J.G. 1976. Latent infections by herpes simplex virus in experimental animals. Surv. Ophthalmol. **21(2)**: 175-177.

Stevens, J.G. 1989. Human herpesviruses: a consideration of the latent state. Microbiol. Rev. **53**:318-332.

Stevens, J.G, and M.L. Cook. 1971. Latent herpes simplex virus in spinal ganglia of mice. Science. **173**:843-845.

Stevens, J.G., E.K. Wagner, G.B. Devi-Rao, M.L. Cook, and L.T. Feldman. 1987. RNA complementary to a herpesvirus α gene mRNA is prominent in latently infected neurons. Science. **235**:1056-1059.

Tedder, D.G., R.D. Everett, K.W. Wilcox, P. Beard, and L.I. Pizer. 1989. ICP4-binding sites in the promoter and coding regions of the herpes simplex virus gD gene contribute to activation of in vitro transcription by ICP4. J. Virol. **63**:2510-2520.

Thomas, M.S., M. Gao, D.M. Knipe, and K.L. Powell. 1992.

Association between the herpes simplex virus major DNA-binding protein and alkaline nuclease. J. Virol. **66**:1152-1161.

Vaughn, P.J., L.M. Banks, D.J.M. Purifoy, and K.L. Powell. 1984. Interactions between herpes simplex virus DNA-binding proteins. J. Gen. Virol. **65**:2033-2041.

Vaughn, P.J., D.J.M. Purifoy, and K.L. Powell. 1985. DNA-binding protein associated with herpes simplex virus DNA polymerase. J. Virol. **53**:501-508.

Vlazny, D.A., A. Kwong, and N. Frenkel. 1982. Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. Proc. Natl. Acad. Sci. USA **79**:1423-1427.

Walz, M.A., R.W. Price, and A.L. Notkins. 1974. Latent ganglionic infection with herpes simplex virus types 1 and 2: viral reactivation in vivo after neurectomy. Science. **184**:1185-1187.

Wang, Y., and J.D. Hall. 1990. Characterization of a major DNA-binding domain in herpes simplex virus type 1 DNA-binding protein (ICP8). J. Virol. **64**:2082-2089.

Watson, R.J., and J.B. Clements. 1980. A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. Nature (London). **285**:329-330.

Weller, S.K. 1991. Genetic analysis of HSV genes required for genome replication, p.105-135. *In* E.K. Wagner (ed), Herpesvirus transcription and its regulation. CRC Press, Boca Raton.

Weller, S.K., E.P. Carmichael, D.J. Goldstein, and L. Zhu. 1988. Use of host range mutants to identify genes involved in DNA replication of herpes simplex virus, p. 53-59. *In* T. Kelly and B. Stillman (ed.), Cancer cells, vol. 6. Eukaryotic DNA replication. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Weller, S.K., K.J. Lee, D.J. Sabourin, and P.A. Schaffer. 1983. Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex type 1 DNA-binding protein. J. Virol. **45**:354-366.

Weller, S.K., M.R. Seghatoleslami, L. Shao, D. Rowse, and E.P. Carmichael. 1990. The herpes simplex virus type 1 alkaline exonuclease is not essential for viral DNA synthesis: isolation and characterization of a *lac*Z insertion mutant. J. Gen. Virol. **71**:2941-2952.

White, D.O, and F.J. Fenner. 1986. Herpesviruses, 401-431. *In* Medical Virology, 3rd edition. Academic Press, Orlando.

Whitley, R.J., S. Spruance, F.G. Hayden, J. Overall, C.A. Alford, Jr, J.M. Gwaltney, Jr, S.-J. Soong, and the NIAID Collaborative Antiviral Study Group. 1984. J. Infec. Dis. **149**:1-8.

Whitley, R.J. 1985. Epidemiology of herpes simplex viruses, p. 1-44. *In* B. Roizman (ed), The herpesvirues, vol.3. Plenum Press, New York.

Whitley, R.J. 1990. Herpes simplex viruses, p. 1843-1887. *In* B.N. Fields, D.M. Knipe, *et al* (ed), Virology, 2nd ed. Raven Press, New York.

Wilcox, K.W., A. Kohn, E. Sklyanskaya, and B. Roizman. 1980. Herpes simplex phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. **33**:167-182.

Wilson, C.M. 1983. Staining of proteins on gels: comparison of dyes and procedures. Methods Enzymol. **91**:236-247.

Wohlenberg, C.R., M.A. Walz, and A.L. Notkins. 1976.

Efficacy of phosphonoacetic acid on herpes simplex virus infection of sensory ganglia. Infec. Immun. **13**:1519-1521.

Wu, C.A., N.J. Nelson, D.J. McGeogh, and M.D. Challberg. 1988. Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. **62**:435-443.

WuDunn, D., and P.G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. -**63**:52-58.

Yamasaki, L., P. Kanda, and R.E. Lanford. 1989. Identification of four nuclear transport signal-binding proteins that interact with diverse transport signals. Mol. Cell. Biol. **9**:3028-3036.

Yoneda, Y., N. Imamoto-Sonobe, Y. Matsuoka, R. Iwamoto, Y. Kiho, and T. Uchida. 1988. Antibodies to asp-asp-glu-asp can inhibit transport of nuclear proteins into the nucleus. Science. **242**:275-278.